Basic Cytotoxicity Detection Kit

catalog #969, trial size (125 tests)

catalog #970, regular size (250 tests)

RESEARCH USE ONLY

1. INTRODUCTION

In a single tube, scientists can now quantify cytolytic killer activity using ImmunoChemistry Technologies' (ICT) Basic Cytotoxicity Kit. It easily separates live target cells from necrotic cells without interference from the effector cells. There is no need to lyse the cells or wait for enzyme release (like LDH, ATP, AK and other assays). This kit does not use any radioisotopes, so it is much safer to run than chromium-51 (^{51}Cr) assays. ICT's Basic Cytotoxicity Kit is the easiest method to calculate cell death.

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[2]. 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[2]. Unfortunately, these techniques have several drawbacks such as: high spontaneous leakage resulting in high backgrounds; high cost; short half-life; and the health risks due to exposure to radioactive material[7].

Flow cytometric assays have been developed to overcome some of the difficulties associated with older assays like lactate dehydrogenase and ^{51}Cr release assays. Radosevic[6] detected NK cytotoxicity activity by staining target cells with the green fluorescent dye, F-18, in combination with the DNA intercalating dye, propidium iodide[6]. 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[1, 3, 4, 8], but the PKH-26 method is still 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. Despite this, following the optimization of a flow cytometric assay, Lee-MacAry[2] compared it with the ^{51}Cr release assay and demonstrated a correlation greater than 95%[2].

Since then, the problems with older flow cytometric assays were overcome when Olin[5] used 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) to stain the membranes of target cells[5]. By staining K562 cells with CFSE, they demonstrated an increase in NK activity following BCG vaccination. Using the same technique to stain *Mycobacterium* infected monocytes, they further demonstrated specific antigen-directed cytolytic activity against *Mycobacterium*.

Building upon the techniques of Olin[5], ICT has improved the flow cytometric assay by combining a membrane stain with a necrosis stain. ICT's Basic Cytotoxicity Kit includes two fluorescent reagents, CFSE and 7-AAD, which enable the flow cytometer to easily differentiate target and effector cell populations for analysis (Figure 7). Traditional enzyme-release assays are often skewed by the large number of necrotic effector cells, but this test is not affected by those cells.

The first of these reagents, CFSE, a green fluorescing membrane stain, is used to confer a green fluorescence feature on the target cell population (Figure 4). The unstained effector cells are then added and incubated with the target cells (referred to as the 'E:T' mixture; Figure 5). Upon completion of the E:T incubation, the second reagent, 7-aminoactinomycin D (7-AAD), a red fluorescing live/dead stain, is added to stain all necrotic cells red by binding to the DNA of membrane-compromised cells (Figure 6).

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 distinguished. 5 control populations are used to compensate the flow cytometer (Figures 7-10). Proper gating of the flow cytometer easily distinguishes between live and necrotic cells within a single sample tube.

CFSE Membrane Stain: excitation at 488 nm; emission in FL-1.

7-AAD Live/Dead Stain: excitation at 488 nm; emission in FL-3.

To include cells in the early stages of apoptosis, use ICT's Total Cytotoxicity & Apoptosis Detection Kit (catalog #972) which includes all the reagents found in the Basic Cytotoxicity Kit plus ICT's SR-FLICA® apoptosis detection reagent. The Total Cytotoxicity & Apoptosis assay was designed to detect cells that do not have a compromised cell membrane, yet have active caspase enzymes. Caspase-positive cells will fluoresce orange-red with SR-FLICA®: these cells are just entering apoptosis, yet are not necrotic.

ICT's Basic Cytotoxicity Kit is the easiest method to assess cytotoxicity.

2. KIT CONTENTS

Catalog #969, trial size, 125 tests, contains:

- 1 vial of CFSE green fluorescing membrane stain, approximately 250 tests, #6162; store at ≤-20°C.
- 1 vial of 7-AAD red fluorescing live/dead vital stain, approximately 125 tests, #6163; store at 2-8°C or frozen.
- 1 bottle of 10X Assay Buffer, 30 mL, #6161; store at 2-8°C or frozen.

Catalog #970, regular size, 250 tests, contains:

- 1 vial of CFSE green fluorescing membrane stain, approximately 250 tests, #6162; store at ≤-20°C.
- 2 vials of 7-AAD red fluorescing live/dead vital stain, approximately 125 tests each, #6163; store at 2-8°C or frozen.
- 1 bottle of 10X Assay Buffer, 60 mL, #685; store at 2-8°C or frozen.

3. STORAGE

The entire kit should be stored frozen however some of the components may be stored refrigerated.

- Store CFSE at ≤-20°C. Use it immediately, or store at ≤-20°C for 6 months protected from light and thawed no more than twice during that time.
- 7-AAD, and 10X Assay Buffer may be stored frozen or refrigerated. Store each unopened component at ≤2-8°C until the expiration date.

4. MSDS

Available online at www.immunochemistry.com.

5. RECOMMENDED MATERIALS

- DMSO, up to 1 mL to reconstitute reagents
- DiH_20, 270-540 mL to dilute 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
- 90% ETOH (in 10% PBS or 1X Assay Buffer) to create live/dead controls for 7-AAD staining
- Hemocytometer
- Centrifuge at <300 x g
- 37°C incubator
- FACS tubes
- 15 mL polystyrene centrifuge tubes
- Ice bath

6. DETECTION EQUIPMENT

Flow Cytometer, 15 mW, 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 membrane stain: excitation at 492 nm; emission at 520-540 nm in FL-1 (Section 13)
- 7-AAD red fluorescing live/dead vital stain: excitation at 546 nm; emission at 647 nm in FL-3 (Section 15)

7. OVERVIEW

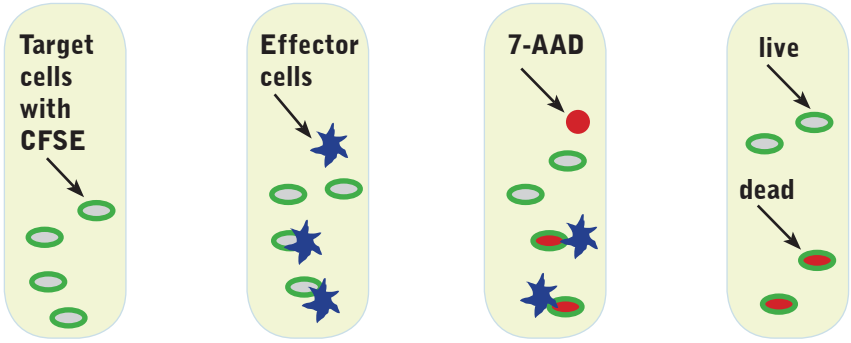
Quantifying cell death with ICT's Basic Cytotoxicity Kit can be completed within a few hours. However, the experiment is performed on living cells, which require periodic maintenance and cultivation several days in advance. Once the proper number of cells has been cultivated, time must be allotted for the experimental treatment or cytotoxic process. Each investigator should adjust the amount of the reagents to use and incubation times to accommodate their particular cell line 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 with your healthy target cells. In addition, several control tubes must be prepared for compensation of the flow cytometer (Section 8).

ICT's Basic Cytotoxicity Kit 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 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 with diH_20 and sterile filter (Section 11).
2. Reconstitute CFSE with 200 µL DMSO (Section 13).
3. Reconstitute 7-AAD with 260 µL DMSO (Section 15).
4. Dilute 2500X CFSE stock 1:250 in sterile 1X Assay Buffer (4 µL into 996 µL).
5. For each sample and control, prepare a 1.8 mL solution of target cells at 1-2 x 10^6 cells/mL in 1X Assay Buffer (Section 12).
6. Prepare control tubes (Section 8).
7. Add 200 µL diluted 10X CFSE to target cells, and all controls except B.
8. Incubate 15 minutes at room temperature.
9. Add 1 mL media, centrifuge, remove supernatant, add 2-3 mL media.
10. Incubate 30 minutes at 37°C.
11. Adjust stained target cells to 2-4 x 10^5 cells/mL and make 200 µL aliquots.
12. Adjust unstained effector cells to the desired concentration.
13. Add 200 µL unstained effector cells to the stained target cells. This forms the 'E:T' mixture at 400 µL.
14. Incubate E:T 4-6 hours at 37°C.
15. Create a positive control of killed cells (Section 9) to generate Control E.
16. Dilute 210X 7-AAD stock 1:10 in sterile 1X Assay Buffer (40 µL into 360 µL).
17. Add 20 µL diluted 21X 7-AAD to Controls D, E, & F.
18. Incubate controls 10 minutes on ice.
19. Run the instrument controls to set up the proper gates and adjust compensation (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 17, Figures 10-12).

FIGURE 1: QUANTITATE CELL DEATH



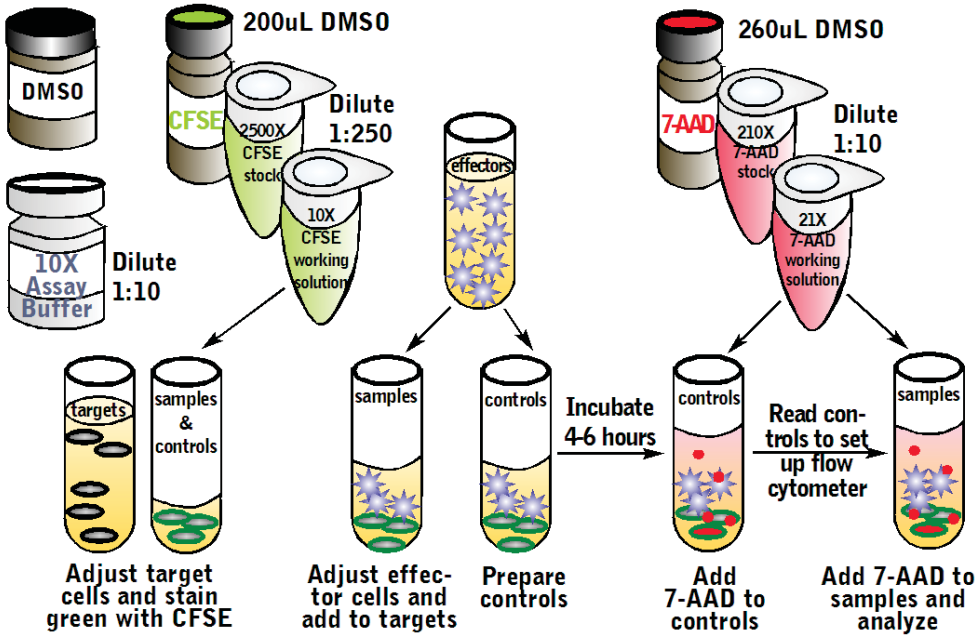
Purify target cells and stain green with CFSE to distinguish them from unstained effector cells during FACS analysis. If studying the effects of a toxic agent, CFSE is optional.

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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8. FLOW CYTOMETRY CONTROLS

Several control populations (Figure 3) are needed to properly gate the flow cytometer and set up compensation to obtain the most accurate results (Section 16). Follow Figures 7-10 to create the control tubes and set up the instrument. Gatings shown here were generated on a BD FACS Caliber; compensation requirements may differ among instruments.

A: A contains target cells stained with CFSE and effector cells. It is used to separate the green target cells from the unstained effector cells in Figure 7. It is also used as a negative control.

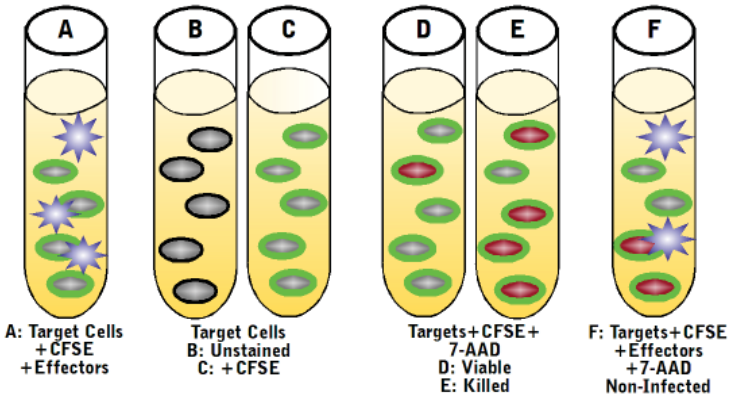
B&C: B contains unstained target cells. C contains target cells stained green with CFSE. They are used to determine the shift of target cells from left to right in Figure 8.

D&E: D and E contain target cells stained green with CFSE and red with 7-AAD. D contains live cells, while the cells in E are killed (Section 9). They will determine the shift of 7-AAD from bottom to top in Figure 9.

Control D will also act as an experimental control to measure the 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. Calculate the percentage of spontaneous cell death and subtract it from the experimental samples. (Figure 9).

F: If the experiment is designed to determine innate or adaptive cytotoxicity using isolated macrophages or monocytes etc., prepare Control F. It contains non-infected target cells stained with CFSE mixed with effector cells to determine spontaneous cell death.

FIGURE 3: INSTRUMENT CONTROLS



9. METHODS TO KILL CELLS

In Section 8, Control E (Figure 9) contains target cells labeled green with CFSE that have been killed and then labeled red with 7-AAD. Here are 2 methods for killing cells:

Method 1: Hot water bath.

1. Immerse the tube of cells in a 56°C water bath for 3-6 minutes, then place on ice.
2. Add 7-AAD to stain necrotic cells (Section 17).

Method 2: Ethanol. However, ethanol may decrease the CFSE membrane stain, so the population may not shift as far to the right (Figure 8).

1. Centrifuge cells at 300 x g for 5 minutes.
2. Carefully remove the supernatant.
3. Add 250 µL of 90-100% ethanol.
4. Vortex 30-60 seconds.
5. Immediately add 1 mL 1X Assay Buffer.
6. Centrifuge at 300 x g for 5 minutes.
7. Carefully remove the supernatant.
8. Add 400 µL media to resuspend cells.
9. Add 7-AAD to stain necrotic cells (Section 17).

FIGURE 4: STAIN TARGET CELLS WITH CFSE

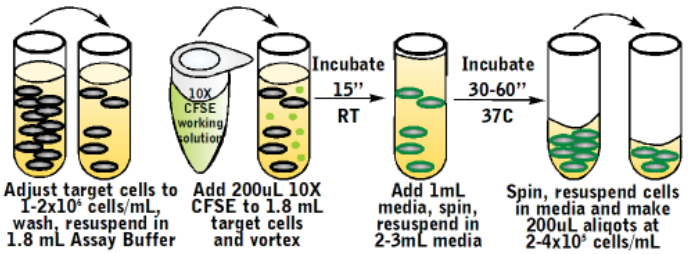


FIGURE 5: ADD EFFECTOR CELLS

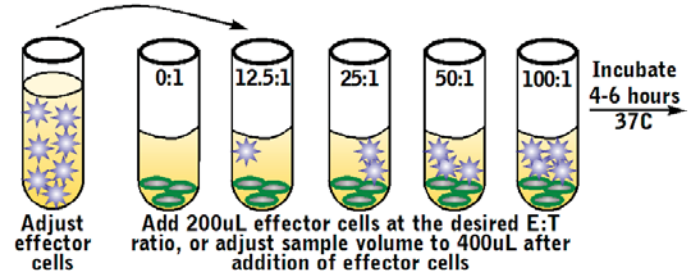
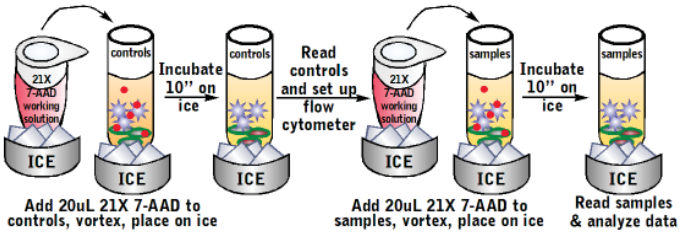


FIGURE 6: LABEL NECROTIC CELLS WITH 7-AAD



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PROTOCOL

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 cells, 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. Proliferation will decrease the average fluorescence intensity of the target cell population. As cell media will quench the CFSE fluorescent signal, the media must be replaced with 1X Assay Buffer before staining with CFSE.

11. DILUTE 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 di$H_2$0 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.

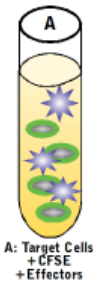
Instead of using 1X Assay Buffer to dilute the reagents, sterile PBS can be used. In some steps, fresh cell culture media can be used in place of Assay Buffer (but not while staining with CFSE). Dispose of excess Assay Buffer by flushing down the sink with water, or by tossing in standard trash bins; see MSDS for further information.

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 di$H_2$0. For example:
   a. Add 30 mL 10X Assay Buffer to 270 mL di$H_2$0 (forming a total volume of 300 mL).
   b. Add 60 mL 10X Assay Buffer to 540 mL di$H_2$0 (600 mL total).
3. Mix for 5 minutes or until all crystals have dissolved.
4. Sterilize by filtration.

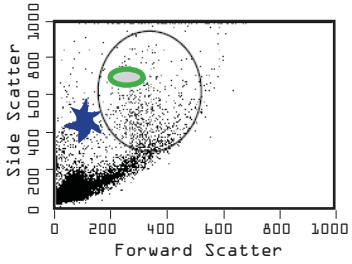
12. ADJUST TARGET CELLS IN ASSAY BUFFER

5. 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 the CFSE fluorescent signal.
6. Wash target cells twice with 1X Assay Buffer to remove any media. Centrifuge at <300 x g for 5 minutes and discard supernatant.
7. Resuspend target cells with 2-3 mL 1X Assay Buffer.
8. Centrifuge at <300 x g for 5 minutes; discard supernatant.
9. Resuspend target cells with 1.8 mL 1X Assay Buffer.

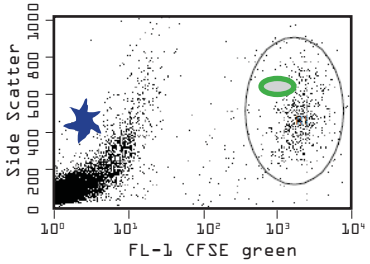
FIGURE 7: CONTROL A WILL DISTINGUISH GREEN CFSE-LABELED TARGET CELLS FROM UNSTAINED EFFECTOR CELLS: FSC VS. SSC AND FL-1 (CFSE) VS. SSC PLOTS



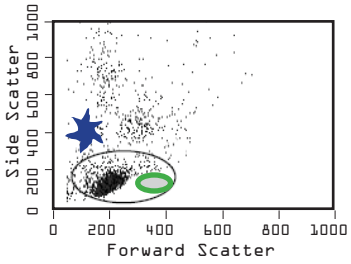
Control A contains CFSE-stained target cells and effector cells (E:T). It is used to distinguish the green CFSE-stained target cells from the unstained effector cells. Run Control A and create a forward scatter (FSC) vs. side scatter (SSC) plot (Figures 7A and 7C). Large cells, like K562 cells (circled) were easy to distinguish from lymphocyte effector cells. Then create a plot of FL-1 (CFSE) 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 (Figures 7C and 7D). Set Control A aside, as it will be used again in Figure 12 {data 121504}



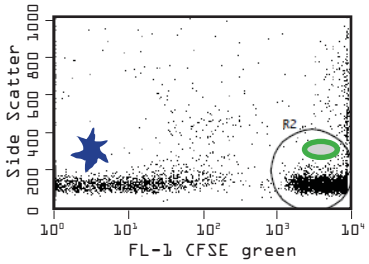
7A: Control A FSC vs SSC



7B: Control A FL-1 vs SSC



7C: *Mycobacterium* FSC vs SSC



7D: *Mycobacterium* FL-1 vs SSC

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13. STAIN TARGET CELLS WITH CFSE

5-(and 6)-carboxyfluorescein diacetate succinimidyl ester, CFSE (catalog #6162), is used to label cell membranes 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.

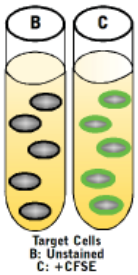
CFSE must first be reconstituted in DMSO, forming a 2500X stock concentrate, and then diluted 1:250 in sterile 1X Assay Buffer to form the 10X working solution that will be used to stain the target cells. Do NOT dilute CFSE in media, as the cell membrane reactive properties of the CFSE stain will be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

10. Reconstitute CFSE with 200 µL DMSO. This yields a 2500X stock concentrate. Mix by swirling or tilting the vial, 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.
11. 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 freeze-thaw cycles, make small aliquots in amber vials or polypropylene tubes.
12. 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 0.996 mL sterile 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 it will quench the fluorescent signal.
13. 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.

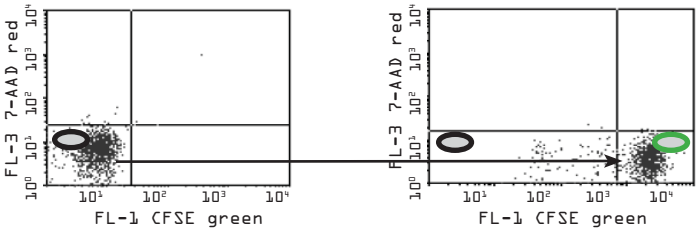
    The optimal concentration of CFSE may vary among cell types. Adjust the concentration of CFSE and the incubation time to adequately stain the target cells for the experiment. Excessive staining may cause problems when compensating the flow cytometer.
14. Incubate 15 minutes at room temperature.
15. Add 1 mL cell culture media to stop the CFSE binding reaction.
16. Centrifuge at <300 x g for 5 minutes; discard supernatant.
17. Resuspend in 2-3 mL cell culture media.
18. Incubate 30-60 minutes at 37°C in a CO_2 incubator. Plan the experiment so the cells incubate no more than 1 hour while setting up the assay.
19. Centrifuge at <300 x g for 5 minutes; discard supernatant.
20. Resuspend with cell culture media. Adjust the concentration of the target cells so that a 200 µL volume will contain 2 x 10^4 target cells.

    For example, start with 1 x 10^5 cells/mL or 2 x 10^4 cells in 200 µL. Too many cells will induce spontaneous cell death. They will be combined with 200 µL of effector cells yielding the desired Effector:Target cell ratio (E:T), such as 50:1 (Section 14).

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



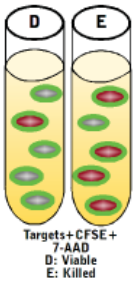
Control B contains unstained target cells. Control C contains CFSE-stained target cells. Run Control B (Figure 8A) and then run Control C (Figure 8B) to compensate for FL-1 (CFSE) vs. FL-3 (7-AAD). When stained green with CFSE (Control C), target cells shift to the right compared to unstained cells (Control 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}.



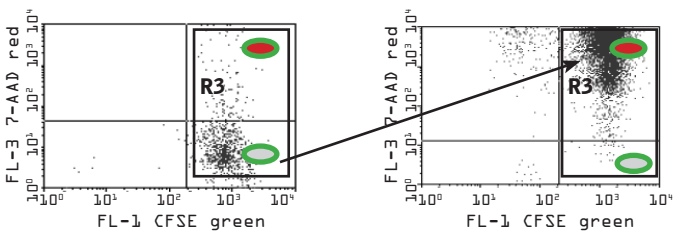
8A: Control B Unstained Target Cells

8B: Control C CFSE-Labeled Green Target Cells

FIGURE 9: CONTROLS D&E WILL DISTINGUISH LIVING FROM DEAD TARGET CELLS AND DETERMINE A BASE LEVEL OF SPONTANEOUS CELL DEATH: FL-1 VS. 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 Control D (Figure 9A) to compensate FL-1 (CFSE) vs. FL-3 (7-AAD). These cells must be viable when compared with the killed cells in Control E. Run Control E and compensate the flow cytometer to ensure that dead cells shift straight up the Y-axis (Figure 9B). 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. 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 {data 072204}.



9A Control D, live target cells

9B Control E, dead target cells

14. ADD EFFECTOR CELLS

21. An optimal E:T cell ratio is required to effectively determine cytolytic activity (Figures 5 and 12). 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, in 400 µL.

    For example, if a 200 µL target cell suspension contains 2 x 10^4 cells (Section 13), add 50 times that number of effector cells (1 x 10^6) in 200 µL. The concentration of effector cells would have to be 5 x 10^6 cells/mL.
22. Add effector cells to samples and Controls A and F and adjust the total volume to 400 µL.

    If the volume is >400 µL when combined, then centrifuge (< 300 x g for 5 minutes) and remove some of the cell culture media in excess of the 400 µL sample size.
23. 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 and 210X stock at ≤-20°C.

24. Reconstitute lyophilized 7-AAD with 260 µL DMSO. This yields a 210X stock concentrate. Mix by swirling or tilting the vial, 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.
25. 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 freeze-thaw cycles, make small aliquots in amber vials or polypropylene tubes.
26. 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. This yields 400 µL of the 21X working solution. Mix by inverting or vortexing the vial. For best results, the 21X working solution should be used within 2 hours, stored on ice, and protected from light.
27. Add 20 µL 21X 7-AAD to Controls D, E, and F just prior to analysis and mix or gently vortex.

    Do not add 7-AAD to the samples until the flow cytometer has been compensated (Section 17).
28. 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.

29. Run Control A (Figure 7) to separate live green fluorescing target cells from unstained effector cells.
30. Run Controls B&C (Figure 8) ensure live green fluorescing target cells fall in the proper region.

FIGURE 10: IDENTIFY 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 FL-1 (CFSE) vs. SSC to identify the green target cells from the unstained effector cells (Figure 7). Create a plot of FL-1 (CFSE) vs. FL-3 (7-AAD) 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 Test is not affected by those cells {data 121504}.

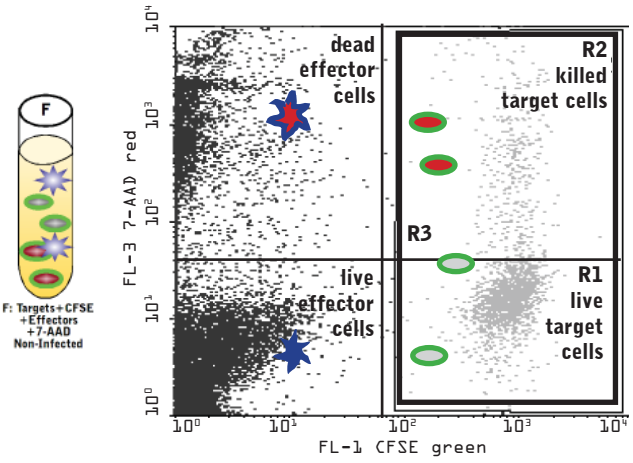


FIGURE 11: CALCULATION OF CYTOTOXICITY

Based on the percentage of green target cells in R3 (Figures 10 and 12) cytotoxicity can be calculated as the precentage 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 Test is the easiest assay to quantify cytotoxicity.

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

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31. Run Controls D&E (Figure 9) 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.
32. 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 to determine spontaneous cell death.

17. LABEL SAMPLES WITH 7-AAD

33. Add 20 µL 21X 7-AAD to the experimental samples (at 400 µL) and mix or gently vortex.
34. Incubate 10 minutes on ice protected from light and analyze as quickly as possible (Section 18).

18. ANALYZE SAMPLES

35. Distinguish green target cells from unstained effector cells using FSC vs. SSC or FL-1 vs. SSC (Figures 7A and 7C).
36. Isolate the target cell population by creating a plot of FL-1 CFSE vs. FL-3 7-AAD (Figures 10 and 12).
37. Create a gate on the green target cell population (R3 in Figures 10 and 12).
38. Count the red and green necrotic target cells (R2 in Figures 10 and 12).
39. Calculate the percentage of cytolytic activity 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).

19. REFERENCES & CITATIONS

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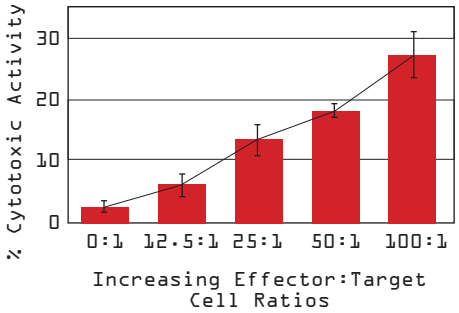
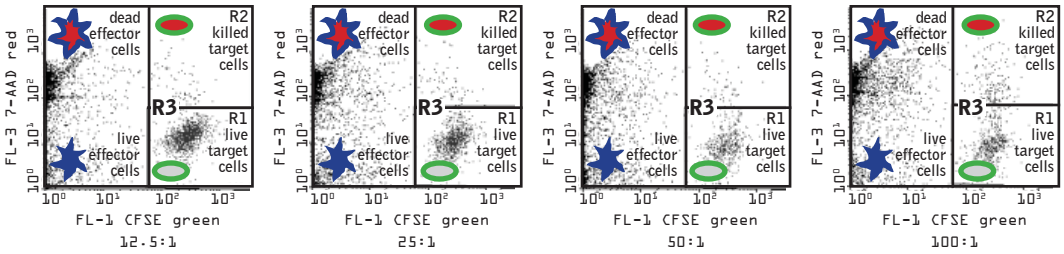
FIGURE 12: EFFECTOR:TARGET CELL RATIOS

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: K563 target cells were treated 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. Cells were analyzed and plotted versus the E:T cell ratio. Cytolytic activity increased to more than 25% as more effector cells were added at 100:1 {data 072204}.





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