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Total Cytotoxicity & Apoptosis Detection Kit

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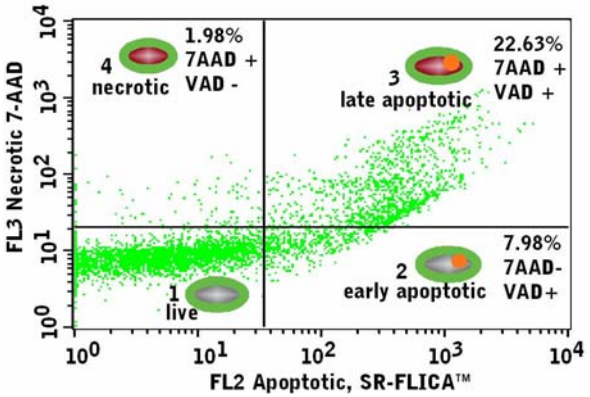

Total Cytotoxicity Flat Manual

For technical questions and orders, please contact us at:

1-800-829-3194
952-888-8788
952-888-8988 fax
www.immunochemistry.com

Cytotoxicity Kit Ordering Information:

	125-test kit	250-test kit
Cytotoxicity Test	catalog# 969	catalog# 970
Total Cytotoxicity Kit	catalog# 971	catalog# 972

Cover, Figure 11: In a single test tube, the Total Cytotoxicity Kit will distinguish the different processes of cellular death:

1) live target cells
2) early apoptotic cells
3) late apoptotic cells
4) necrotic cells

Cells in early apoptosis are not detectable by any other method.

 Publication Version: Total Cytotoxicity Flat Manual #823-021306. Written by Dr. Michael Olin and Sally A. Hed, with assistance from Dr. Brian W. Lee and Erin M. Christey. Graphics by Sally A. Hed and Erin M. Clark.

www.immunochemistry.com
Immunochemistry Technologies, LLC
9401 James Avenue South, Suite 155
Bloomington, MN 55431
800-829-3194 / 952-888-8788
fax 952-888-8988

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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 accomplished 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 chromium-51 (^{51}Cr) from lysed target cells (1). However, these techniques have several drawbacks such as: high spontaneous leakage resulting in high backgrounds; high cost; short half-life; the potential health risk due to radioactive material (2); and the inability to detect early-stage apoptotic cells.

Flow cytometric assays have been developed to overcome some of the difficulties with lactate dehydrogenase and ^{51}Cr release assays. Radosevic (1990) detected 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), 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 cytometer assay, Lee-MacAry (2001) compared it with the ^{51}Cr release assay and demonstrated a correlation greater than 95% (1).

Since then, the problems with older flow cytometric assays were overcome when Olin (2005) used 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) to stain the membranes of target cells (8). 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 (2005), Immunochemistry Technologies, LLC (ICT) has improved the flow cytometric assay by combining it with ICT's FLICA™ apoptosis detection reagents to concurrently quantify caspase-positive cells as well. Now, in a single tube, scientists can differentiate cytolytic killer activity from apoptosis with ICT's Total Cytotoxicity Kit. There is no need to lyse the cells or wait for enzyme release (like LDH, ATP, AK and other assays). Since this kit does not use any radioisotopes, it is much safer to run than the ^{51}Cr assays. At least 10% more cytolytic activity is often measured when apoptotic cells are also identified.

ICT's Total Cytotoxicity Kit includes three fluorescent reagents, which enable the flow cytometer to easily separate the target and effector cell populations for

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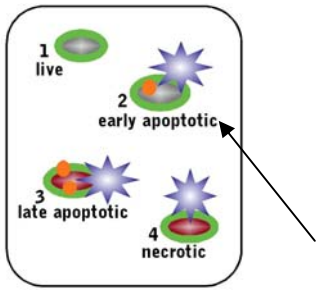
analysis. The first of these reagents, CFSE, a green membrane stain, is used to label all the target cells green. The unstained effector cells are then added and incubated with the target cells (E+T).

Apoptotic target cells can be identified by staining with ICT's reagent, SR-FLICA™ (SR-VAD-FMK, a red fluorescent caspase inhibitor), which binds to active apoptosis up-regulated caspase enzymes (9). Upon completion of the E+T incubation period (which includes exposure to the apoptosis detection probe), ICT's reagent, 7-aminoactinomycin D (7-AAD), a red live/dead stain, is added to stain all dead cells red by binding to the DNA of membrane-compromised cells.

Because 7-AAD will not detect cells in the early stages of apoptosis, it is important to expose the target cells to SR-FLICA™. This test will often reveal a significant percentage of cells that were 7-AAD-negative (indicating they are alive) yet caspase positive (apoptotic and dying). These cells cannot be detected by any other method. Careful gating of SR-FLICA™ and 7-AAD fluorophores (using the FL2 and FL3 channels respectively), distinguishes between the red 7-AAD live/dead fluorescence signal and the red SR-FLICA™ caspase-specific signal within a single sample tube.

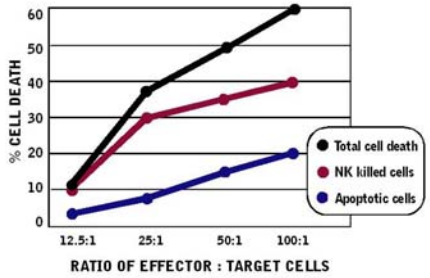


In a single test tube, the Total Cytotoxicity Kit will distinguish four stages of cellular death:

1) live cells
2) early apoptotic
3) late apoptotic
4) necrotic

Cells in early apoptosis are not detectable by any other method.

As all of the target cells are initially labeled with CFSE membrane stain, and the effector cells are not, the two populations can be easily distinguished. The percentage of cytotoxicity is calculated by creating a live/dead vs. apoptosis graph gated from the target population (cover, Figure 11) and adding percentages of 7-AAD$^+$, 7-AAD$^+$ VAD$^+$, and VAD$^+$ quadrants.



The true level of cytotoxicity is revealed by including early apoptotic cells.

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2. Contents of the 125-test Total Cytotoxicity Kit, Catalog# 971

- 1 amber vial of lyophilized green membrane stain, CFSE, 250 tests per vial, catalog# 6162
- 1 amber vial of lyophilized red live/dead stain, 7-AAD, 125 tests per vial, catalog# 6163
- 1 amber vial of lyophilized SR-FLICA™ poly caspases apoptosis detection reagent, SR-VAD-FMK, 125 tests per vial, catalog# 6221
- 1 bottle of 30 mL 10X Assay Buffer, catalog# 6161
- Assay Manual, catalog# 824
- MSDS sheets

3. Contents of the 250-test Total Cytotoxicity Kit, Catalog# 972

- 1 amber vial of lyophilized membrane stain, CFSE, 250 tests per vial, catalog# 6162
- 2 amber vials of lyophilized red live/dead stain, 7-AAD, 125 tests per vial, catalog# 6163
- 2 amber vials of lyophilized SR-FLICA™ poly caspases apoptosis detection reagent, SR-VAD-FMK, 125 tests per vial, catalog# 6221
- 1 bottle of 60 mL 10X Assay Buffer, catalog# 685
- Assay Manual, catalog# 824
- MSDS sheets

4. Storage and Shelf-Life

- Store the CFSE ≤-20°C until ready to use and protect from light.
- Store the rest of the kit components at 2 - 8°C.
- Once reconstituted, the stock concentrates should be used immediately or stored at ≤-20°C protected from light.
- Once diluted, store the 1X assay buffer at 2 - 8° C for up to 14 days.
- Additional assay buffer may be ordered by calling ICT at 1-800-829-3194 or 952-888-8788.

5. Safety Information

- Use gloves while handling CFSE, 7-AAD, SR-FLICA™, and assay buffer.
- Dispose of all liquid components down the sink and flush with copious amounts of water. Solid components may be tossed in standard trash bins.
- MSDS sheets are available at www.immunochemistry.com/MSDS.htm or by calling 1-800-829-3194 or 952-888-8788.

6. Recommended Materials and Equipment (not all are required)

- FACS tubes
- Cultured cells with media and tissue culture materials
- 15 mL polystyrene centrifuge tubes
- Amber vials or polypropylene tubes for storage of the stock solutions at ≤-20°C, if aliquoted
- Centrifuge at <300 x g
- 37°C CO_2 incubator
- Vortexer

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- Pipette(s) capable of dispensing at 10µL, 50µL, 200µL, 300µL, 1mL
- Sterile/endotoxin-free DIH_2O
- Sterile filter
- Dimethyl sulfoxide (DMSO)
- Ice bath

7. Flow Cytometer, 15 mW, 488 nm Argon Excitation Laser

- CFSE green membrane stain: excitation at 488nm; emission in FL1
- SR-FLICA™ caspase detection stain at 488 nm; emission in FL2
- 7-AAD live/dead stain: excitation at 488 nm; emission in FL3
- 8 control tubes (section 18)

8. Overview of the Total Cytotoxicity Assay

Labeling cells with ICT's Total Cytotoxicity Kit can be completed within a few hours. However, the Total Cytotoxicity Kit is used with 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 your experimental manipulation of the cells.

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. Several control tubes must be prepared for compensation of the flow cytometer, which can be made during the incubation period.

As the 10X CFSE and 21X 7-AAD working solutions must be used immediately, these reagents should be prepared just prior to use and handled under low-light conditions. Here is a quick overview:

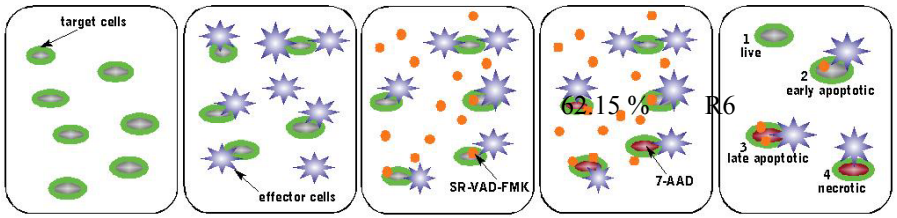

Figure 1: Overview of the assay.

1. Dilute 10x Assay Buffer with DI H_2O.
2. Reconstitute CFSE with 200 µL DMSO.
3. Reconstitute SR-FLICA™ with 100 µL DMSO.
4. Reconstitute 7-AAD with 260 µL DMSO.
5. Further dilute the CFSE stock 1:250 in sterile assay buffer (4 µL into 996 µL).

6. Prepare a 1.8 mL solution of target cells at ~1-2 x 10^6 in 1x assay buffer.
7. Prepare 8 control tubes (section 18).
8. Add 200 µL of 10x CFSE to target cells, and all controls except B.
9. Incubate ~15 minutes at RT.
10. Add 1 mL media, centrifuge, remove supernatant, add 2-3 mL media.
11. Incubate at 37°C for ~30 minutes.
12. Adjust 100 µL stained target cells to 2-4 x 10^4.
13. Add 100 µL unstained effector cells (adjusted to the desired concentration) to the stained target cells (E+T).
14. Incubate the E+T mixture 3.25-5.25 hours at 37°C.
15. Further dilute the SR-FLICA™ stock 1:12.6 in media (10 µL into 116 µL).
16. Add 10 µL of 20x SR-FLICA™ to the E+T mixture and controls D, E, & H.
17. Incubate 45 minutes at 37°C.
18. Add 200 µL RPMI and place cells on ice.
19. Further dilute 7-AAD 1:10 in sterile assay buffer (40 µL into 360 µL).
20. Add 20 µL of 21x 7-AAD to controls F, G, & H (do not add 7-AAD to samples until the flow cytometer has been compensated).
21. Incubate controls 10 minutes on ice.
22. Set up the proper instrument gating and make compensation adjustments based on controls (section 18).
23. Add 20 µL of 21x 7-AAD to samples.
24. Incubate samples 10 minutes on ice.
25. Read and analyze (section 19).

9. Calculation Worksheets

To help calculate the required amount of target and effector cells, ICT has created an online Excel document. Download it from the ICT website at www.immunochemistry.com. The spreadsheet is linked between two worksheets; the first worksheet will calculate target cells, and the second worksheet will calculate effector cells.

10. Preparation of 1X Assay Buffer

The assay buffer is used to 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 refrigeration). It is supplied as a 10X concentrate which must be diluted to 1X with **sterile/endotoxin-free** DI H_2O prior to use and sterile filtered.

Instead of using the assay buffer to dilute the reagents, sterile 10X PBS can be used. When washing the cells, fresh cell culture media can be used in place of the assay buffer. If more assay buffer is needed, please contact ICT at 1-800-829-3194 or 952-888-8788 to order another bottle. Dispose of excess wash buffer by flushing down the sink with copious amounts of water or by tossing in standard trash bins; see MSDS for further information.

1. If necessary, gently warm the 10X concentrate to completely dissolve any salt crystals that may have come out of solution. Do not let it boil.

a. For the 125-test kit, add the entire bottle (30 mL, catalog# 6161) to 270 mL sterile/endotoxin-free DI H_2O (to make 300 mL).
b. Or, for the 250-test kit, add the entire bottle (60 mL, catalog# 685) to 540 mL sterile/endotoxin-free DI H_2O (to make 600 mL).
c. Or, if not using the entire bottle, dilute it 1:10 in sterile/endotoxin-free DI H_2O. For example, add 10 mL 10X assay buffer to 90 mL sterile/endotoxin-free DI H_2O (to make 100 mL).

2. Let the solution stir for 5 minutes or until all crystals have dissolved.
3. Sterilize by filtration.
4. If not using the 1X assay buffer the same day it was prepared, store it covered at 2 - 8°C for up to 14 days (it does not contain any preservatives).

11. Reconstitution and Storage of CFSE Membrane Stain

5-(and 6)-carboxyfluorescein diacetate succinimidyl ester, CFSE, is used to stain cell membranes green. In this assay, it is used to label all target cells green prior to exposure to the effector cells. The CFSE reagent 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 2500X CFSE stock concentrate, and then diluted 1:250 in **sterile** assay buffer to form a final 10X CFSE working solution. For best results, the diluted 10X CFSE working solution should be prepared immediately prior to use; however, the reconstituted 2500X CFSE stock concentrate can be stored at ≤-20°C for future use.

- **Store CFSE at ≤-20˚C.**
- **The newly reconstituted 2500X CFSE stock concentrate must be used or frozen at ≤-20˚C immediately after it is prepared and protected from light during handling.**

1. Reconstitute each vial of lyophilized CFSE (catalog #6162) with 200 µL DMSO. This yields a 2500X stock concentrate. (Each kit contains 1 vial.)
   a. 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 (RT), this should take just a few minutes.
2. If immediately using the 2500X CFSE stock, proceed to section 12.
3. If not using all of the 2500X CFSE stock concentrate at the same time it is reconstituted, it may be stored at ≤-20°C for 6 months.
   a. Make small aliquots of 20-40 µL in amber vials or polypropylene tubes and store at ≤-20°C protected from light. Avoid freeze-thaw cycles.

12. Reconstitution and Storage of Sulforhodamine FLICA™ Poly Caspases Apoptosis Detection Reagent (SR-VAD-FMK)

Cells that are in the early stages of apoptosis can be detected by ICT's **F**luorochrome **L**abeled **I**nhibitors of **CA**spases probes (FLICA™). These probes are cell membrane permeant and form a covalent bond with active caspase enzymes in cells undergoing apoptosis (9). ICT's red poly caspases FLICA™ probe, sulforhodamine-labeled Val-Ala-Asp-fluoromethyl ketone (SR-VAD-FMK), has been optimized for this kit. Just add the reagent to the mixture of target and

effector cells, and caspase-positive cells will fluoresce red. Because this reagent is always fluorescent, an optional set of wash steps may be necessary to remove any unbound SR-FLICA™ probe from the cells.

Because 7-AAD may not detect cells in the early stages of apoptosis, it is important to expose the target cells to SR-FLICA™. Careful gating of SR-FLICA™ and 7-AAD fluorophores (using the FL2 and FL3 channels respectively), distinguishes the 7-AAD live/dead fluorescence signal and the red SR-FLICA™ caspase-specific signal within a single sample tube. This test will often reveal a significant percentage of early apoptotic cells that were 7-AAD-negative (indicating they are alive) yet caspase positive (apoptotic). These early-apoptotic soon-to-be-7-AAD-positive cells can then be included in the overall percentage of total cell death.

The SR-FLICA™ reagent 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 252X SR-FLICA™ stock concentrate, and then diluted 1:12.6 in sterile assay buffer (or RPMI) to form a final 20X SR-FLICA™ working solution. For best results, the 20X SR-FLICA™ working solution should be prepared immediately prior to when it is to be used; however, the reconstituted 252X SR-FLICA™ stock concentrate can be stored at ≤-20°C for future use.

- **Store SR-FLICA™at ≤-20˚C.**
- **The newly reconstituted 252X SR-FLICA™stock concentrate must be used or frozen the same day it is prepared and protected from light during handling.**

1. Reconstitute each vial of lyophilized SR-VAD-FMK (catalog #6221) with 100 µL DMSO. This yields a 252X stock concentrate. (The 125-test kit contains 1 vial; the 250-test kit contains 2 vials.)
   a. 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 (RT), the reagent should be dissolved within a few minutes.
2. If immediately using all the 252X SR-FLICA™ stock, proceed to section 13.
   a. If not using all reagents, make small aliquots of 20-40 µL in amber vials or polypropylene tubes and store at ≤-20°C protected from light. Avoid freeze-thaw cycles.

13. Reconstitution and Storage of 7-AAD Live/Dead DNA Stain

7-aminoactinomycin D (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. This dye will penetrate the structurally compromised cell membranes of dead and dying cells and complex with DNA. Because 7-AAD may not stain cells in the early stages of apoptosis, ICT has included SR-FLICA™ to concurrently detect apoptotic cells with this assay. The intercalated 7-AAD dye exhibits a red fluorescence in the FL3 region with maximum output at 647 nm.

The 7-AAD reagent 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 7-AAD stock concentrate, and then diluted 1:10 in sterile assay buffer to form a final 21X 7-AAD working solution. For best results, the 21X 7-AAD working solution should be prepared immediately prior to use; however, the reconstituted 210X 7-AAD stock concentrate can be stored at ≤-20°C for future use.

- **Store 7-AAD at ≤-20˚C.**
- **The newly reconstituted 210X 7-AAD stock concentrate must be used or frozen at ≤-20˚C immediately after it is prepared and protected from light during handling.**

1. Reconstitute each vial of lyophilized 7-AAD (catalog #6163) with 260 µL DMSO. This yields a 210X stock concentrate. (The 125-test kit contains 1 vial; the 250-test kit contains 2 vials.)
   a. 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 (RT), this reagent should be dissolved within a few minutes.
2. If immediately using the 210X 7-AAD stock, proceed to section 14.
3. If not all of the 210X 7-AAD stock concentrate will be used the same time it is reconstituted, the unused portion may be stored at ≤-20°C for 6 months.
   a. Make small aliquots of 20-40 µL in amber vials or polypropylene tubes and store at ≤-20°C protected from light. Avoid freeze-thaw cycles.

14. Target Cell Staining

All target cells must be stained green with CFSE to distinguish them from non-stained effector cells. Do not use target cells that are capable of proliferating more then 4 hours prior to assay. Proliferation will decrease the average fluorescent intensity of the target cell population. For help calculating the level of target and effector cells needed, download ICT's cytotoxicity worksheet from www.immunochemistry.com.

1. Dilute the 2500X CFSE stock (section 11) 1:250 in **sterile** assay buffer. For example, add 4 µL of the 2500X CFSE stock to 0.996 mL **sterile** assay buffer and mix. This yields 1 mL of 10X CFSE working solution.
2. Adjust target cells to 1-2 x 10^6 cells in 1.0 mL 1X assay buffer.
3. Wash target cells 2 times with 1X assay buffer.
4. Resuspend target cells in 1.8 mL of 1X assay buffer.
5. Add 200 µL of 10X CFSE working solution to each 1.8 mL suspension of target cells and gently vortex. Using less volume may increase difficulty in compensation of the flow cytometer. Add to all control tubes except B.
6. Incubate tubes 15 minutes at room temperature (incubation time may be increased for brighter staining).
7. Add 1 mL cell culture media to stop the CFSE binding reaction.
8. Centrifuge and discard supernatants.
9. Resuspend CFSE-labeled target cells in 2-3 mL cell culture media.
10. Incubate at 37°C for 30 - 60 minutes in a CO_2 incubator (plan your experiment so the cells incubate no more than an hour while setting up the assay).

11. Adjust the concentration of the target cells to a total volume of 100 µL in cell culture media. We recommend starting with 2 - 4 x 10^4 cells, as too many cells will induce spontaneous cell death.

- **The optimal concentration of CFSE needed may vary depending on the type of cells used. Each investigator should adjust the CFSE membrane staining conditions to adequately stain the type of target cells used in their particular experimental model (Figure 5).**

15. Effector Cell Addition

1. Adjust effector cells to the desired Effector/Target cell ratio (such as 50:1) and add to appropriate sample and control tubes.
2. Adjust the volume of each sample and control tube to 200 µL.
3. Incubate 4 - 6 hours (incubation time may vary depending on your experimental design).
4. For the last 45 minutes of this incubation, add the 20x SR-FLICA™ reagent (section 16).

- **An optimal effector/target cell ratio is required to effectively determine cytolytic activity. For example, K562 target cells were stained with CFSE and adjusted to 1.5 x 10^4 cells/tube. Effector cells were added at final effector/target cell ratios of 0:1, 12.5:1, 25:1, 50:1, and 100:1. Cells were incubated for 4 hours to allow the cytolytic activity to progress. They were subsequently analyzed for CTL activity and plotted versus the effector/target cell ratio (Figure 6).**

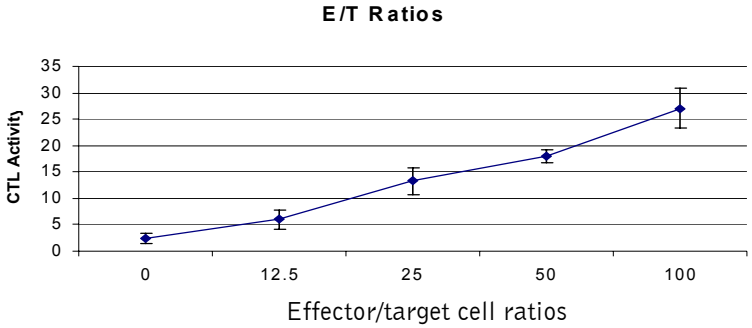

Figure 2: CTL activity increased when more effector cells were used.

- **When using this assay for the first time, optimize the ratio of effector to target cells needed for your particular cell line by performing a titration experiment.**
- **Also optimize the incubation period for your particular cell line and experimental conditions by setting up several samples to incubate for different lengths of time.**

16. Detection of Early & Late Apoptotic Cells with SR-FLICA™

1. Dilute the 252X SR-FLICA™ stock 1:12.6 in media. For example, add 10 µL of the 252X SR-FLICA™ stock to 116 µL media. This yields 126 µL of the 20X working solution. Mix by inverting or vortexing the vial at RT.
2. Add 10 µL of 20X SR-FLICA™ working solution to controls D, E, and H and sample tubes (at 200 µL) 45 minutes prior to termination of E/T incubation (section 12).
3. Carefully mix the SR-FLICA™ probe and incubate for the remaining 45 minutes at 37° C, protected from light.
4. Following incubation, add 200 µL RPMI and place on ice.

17. Detection of Necrotic Cells with 7-AAD

Dead target cells resulting from cytolytic activity of the effector cells can be identified and quantitated using the vital stain 7-aminoactinomycin D (7-AAD). This dye will penetrate structurally compromised cell membranes of dead and dying cells and complex with DNA. 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. The intercalated 7-AAD dye fluoresces red in the FL3 region with maximum output at 647 nm.

1. Dilute the 210X 7-AAD stock 1:10 in **sterile** assay buffer.
   a. For example, add 40 µL of the 210X 7-AAD stock to 360 µL **sterile** assay buffer. This yields 400 µL of the 21X 7-AAD working solution.

- **The 21X 7-AAD working solution must be used the same time it is prepared.**

2. Set up the proper instrument gating and compensation adjustments based on the control cells. Add 20 µL 7-AAD to controls F, G, and H just prior to analysis. (Once the instrument is set up, add 7-AAD to samples.)
3. Mix or gently vortex controls and place in an ice bath.
4. Incubate for 10 minutes protected from light.
5. Set up the instrument.
6. Add 20 µL of the 21X 7-AAD working solution to the sample tubes.
7. Gently vortex.
8. Incubate on ice for 10 minutes and analyze as quickly as possible.
9. When running your sample tubes after the controls have been run and compensation set, first gate the CFSE-stained target cells (FL1, R3 in Figure 9). From within this pool of target cells, prepare a dot plot of SR-FLICA™ (FL2) vs. 7-AAD (FL3) (Figure 10) to identify the four stages of cellular death:
   1. Live target cells (CFSE-stained)
   2. Early apoptotic cells (CFSE and SR-FLICA™ stained)
   3. Late apoptotic cells (CFSE, SR-FLICA™ stained, 7-AAD stained)
   4. Dead cells (7-AAD stained).

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18. Required Controls for Flow Cytometry Set Up

Proper compensation of the instrument is required to obtain accurate results. Compensation requirements may differ among instruments. Use control tubes as shown in Figure 3 in order to properly:

1. Separate target cells from effector cells (control tube A, Figure 4)
2. Determine the shift of target cells stained with CFSE from left to right (control tubes B & C, Figure 5)
3. Determine the shift of SR-FLICA™ from left to right (control tubes D & E, Figure 6)
4. Determine the shift of 7-AAD from bottom to top (control tubes F & G, Figure 7).

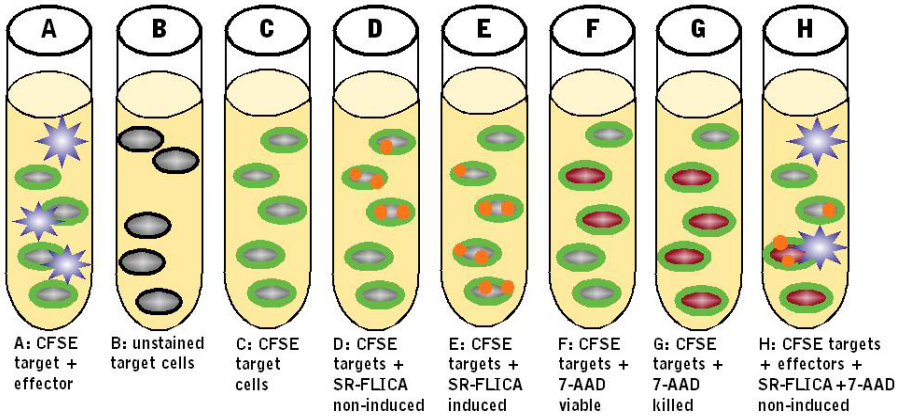

Figure 3: 8 control tubes are needed for instrument set-up.

Control A: Using a control tube containing CFSE-stained target cells and effector cells (E+T), create a forward scatter (FSC) vs. side scatter (SSC) plot (Figure 4a). Large cells, like K562 cells (circled) are easy to distinguish from lymphocyte effector cells. Also create a FL1 (CFSE) vs. SSC scatter plot (Figure 4b). This plot becomes particularly important when gating on target cells that are the same size as effector cells. Create 2 tubes, or rerun as in Figure 8.

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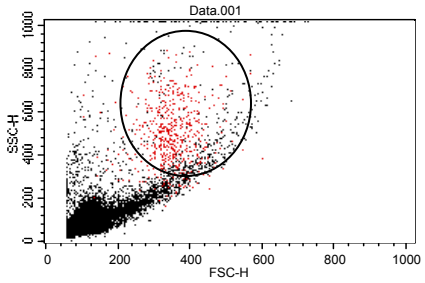

Figure 4a: Control A, FSC vs. SSC

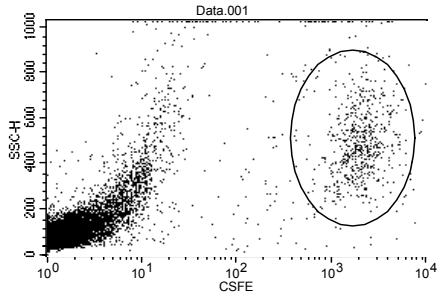

Figure 4b: Control A, FL1 vs. SSC

Control B: Run a control of target cells (unstained) to set up the initial flow compensation for FL1 (CFSE) vs. FL3 (7-AAD), Figure 5a.

Control C: Run a control of CFSE-stained target cells to compensate for FL3 (7-AAD) vs. FL1 (CFSE), Figure 5b. Compare with unstained cells, Control B. Adjust the PMT voltage so that the stained target cells population falls within the 3rd or 4th decade (here, the cells shift to the right). Save the data to ensure that target cells are properly gated during analysis.

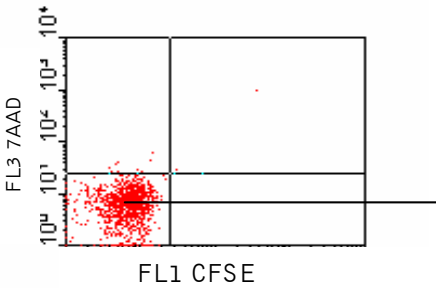

Figure 5a: Control B, unstained.

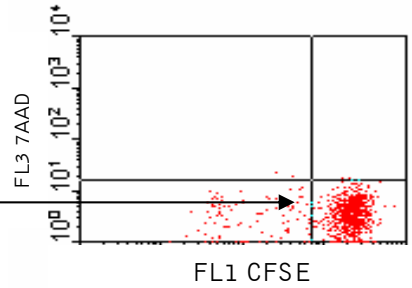

Figure 5b: Control C, CFSE-stained.

Control D: Run a non-induced, CFSE and SR-FLICA™ dually-stained target cell control tube to properly compensate FL2 SR-FLICA™ vs. FL3 7-AAD, Figure 6a. Depending upon your cell line, a small percentage of the negative control population will naturally undergo apoptosis and appear to the right.

Control E: If possible, use an apoptosis-induced, CFSE and SR-FLICA™ dually-stained target cell control tube to properly compensate FL2 SR-FLICA™ vs. FL3 7-AAD, Figure 6b. Compared with non-induced cells (Control D, Figure 6a), apoptotic cells will shift to the right (not all cells will undergo apoptosis).

Total Cytotoxicity Flat Manual

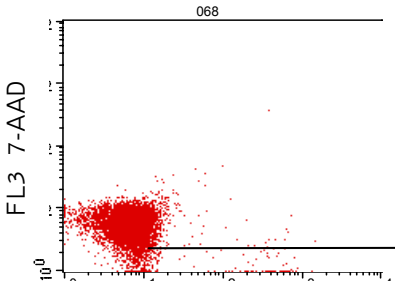

Figure 6a: Control D, non-induced CFSE and SR-FLICA™ stained cells.

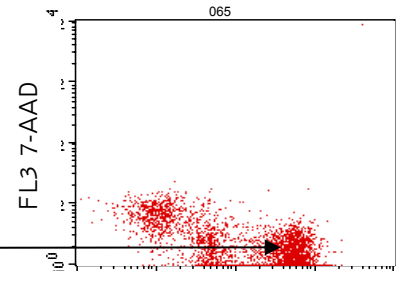

Figure 6b: Control E, apoptosis-induced CFSE and SR-FLICA stained cells used to set up the instrument.

Control F: Run a tube of CFSE-stained target cells dually stained with 7-AAD to compensate FL1 (CFSE) vs. FL3 (7-AAD), Figure 7a. These cells must be viable: compare with killed cells (Control G, Figure 7b) to finish compensating the flow cytometer to ensure that dead cells shift straight up the Y-axis. This control will also determine the level of spontaneous cell death that normally occurs within your cell line.

Control G: Run a tube of killed CFSE-stained target cells dually stained with 7-AAD, Figure 7b. This control demonstrates that killed 7-AAD-positive cells migrate up into the higher red fluorescence output region of the dot plot. Compare with live cells from Control F, Figure 7a.

a. CFSE-stained target cells can be killed in a hot water bath.
   i. Immerse the tube of cells for 3 - 6 minutes in a 56˚C water bath.
   ii. Place on ice.
   iii. Add 7-AAD to stain the dead cells (section 17).
b. Alternatively, CFSE-stained target cells can be killed with ethanol. However, ethanol may decrease the CFSE membrane stain, shifting the population to the left.
   i. Centrifuge the cells at 300 x g for 5 minutes.
   ii. Carefully remove the supernatant by aspiration or blotting the test tube onto paper towels.
   iii. Add 250 µL of 90-100% ethanol.
   iv. Vortex 30 - 60 seconds.
   v. Immediately add 1 mL of 1X wash buffer.
   vi. Centrifuge at 300 x g for 5 minutes.
   vii. Carefully remove the supernatant.
   viii. Resuspend the cells in 400 µL cell media.
   ix. Add 7-AAD to stain the dead cells (section 17).

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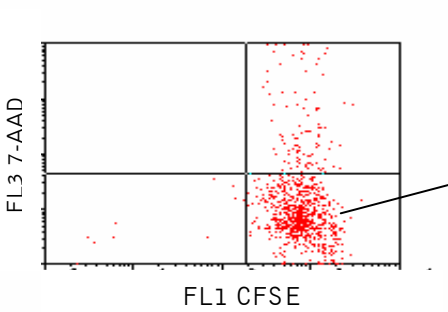


Figure 7a: Control F, live CFSE-stained target cells dually stained with 7-AAD live/dead stain.

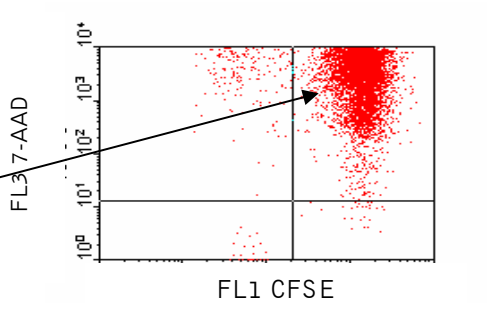


Figure 7b: Control G, killed CFSE-stained target cells dually stained with 7-AAD live/dead stain.

Control A (repeat): Using the compensation set up for Figure 7a, draw a gate around the CFSE and 7-AAD positive target cells (Figure 7c).

- **Set acquisition to collect events within R3.**

Create dot plot of FL2 SR-FLICA™ vs. FL3 7-AAD gated off the region R3. Rerun control A (CFSE-stained target cells mixed with effector cells) as a negative control to ensure proper compensation for the machine (Figure 8). Since this control has not been stained with 7-AAD or SR-FLICA, there should be very little background.

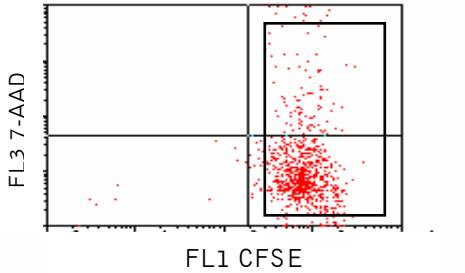


Figure 7c: Control F, live CFSE-stained target cells dually stained with 7-AAD live/dead stain. Cells on top-right are green and red; cells on bottom are green.

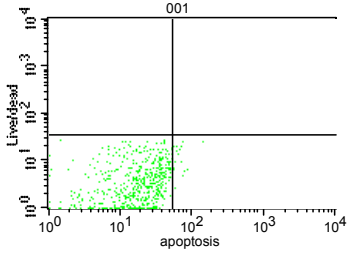


Figure 8: Rerun negative control A FL2 vs. FL3 to ensure proper compensation.

Control H: Run a control of CFSE-stained target cells (non-induced) with effector cells and stained with SR-FLICA™ and 7-AAD, Figure 9. You may need to readjust quadrant; create negative and positive populations quadrant cut off based. This control will also reveal the levels of spontaneous death and apoptosis in your non-induced cells.

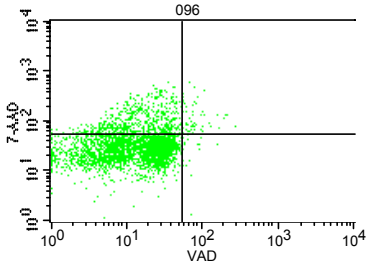


Figure 9: Control H, non-induced target cells combined with effector cells and stained with everything (FL2 vs. FL3).

19. Sample Data Analysis

1. Once you have run the control tubes and set compensation for the instrument, stain the samples with 7-AAD on ice, incubate 10 minutes, and analyze.
2. From the sample data collected, derive a FSC vs. SSC plot or FL1 (CFSE) vs. SSC to separate the target population from the effector cells (like Figure 2).
3. From the sample data, create a plot of FL1 (CFSE) vs. FL3 (7-AAD), and create a gate on the target cell population, R3 (R1+R2=R3) (Figure 10).
4. Gating from R3, derive a plot of the poly caspases apoptosis reagent in FL2 (SR-FLICA™) vs. live/dead viability stain in FL3 (7-AAD) (Figure 11). This will allow researchers to include all apoptotic events in their analysis of total cytotoxicity based on the percentage of cells in each quadrant, leading to more accurate results (Figure 12).

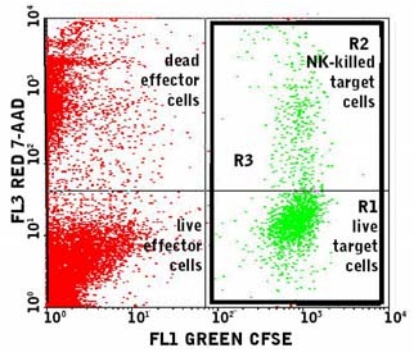
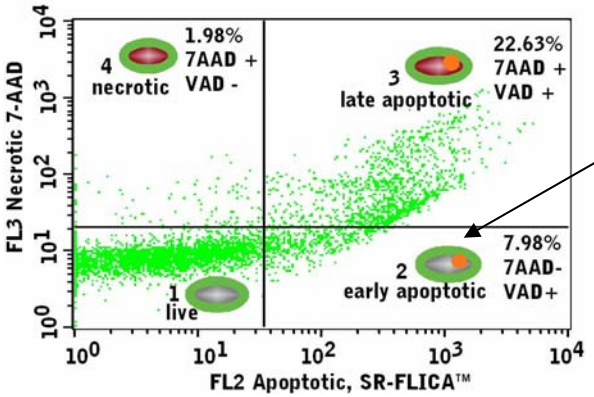


Figure 10: Identify all target cells and gate R1 and R2 as R3.

In a single test tube, the Total Cytotoxicity Kit will identify and distinguish four stages of cellular death.



Cells in early apoptosis are not detectable by any other method.

Figure 11: A dot plot of FL2 (SR-FLICA™) vs. FL3 (7-AAD) reveals 4 populations of cells:

1. Lower left: Apoptosis-negative and live/dead-negative cells (viable live cells).
2. Lower right: Apoptosis-positive and live/dead-negative cells. These cells are in early apoptosis,and are not detectable by other methods.
3. Upper right: Apoptosis-positive and live/dead-positive cells (late apoptotic cells).
4. Upper left: Apoptosis-negative and live/dead-positive cells (necrotic cells).

Total Cytotoxicity = ∑ (quadrants 2+3+4)

eg. ∑ (7.98+22.63+1.98)= 32.59%

Figure 12: Using this formula, total cell death can be calculated and graphed.

20. References

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Immunochemistry Technologies, LLC
9401 James Avenue South, Suite 155
Bloomington, MN 55431
800-829-3194 / 952-888-8788
fax 952-888-8988
www.immunochemistry.com