Total Cytotoxicity & Apoptosis Detection Kit

catalog #971, trial size (125 tests)

catalog #972, regular size (250 tests)

RESEARCH USE ONLY

1. INTRODUCTION

In a single tube, scientists can now differentiate cytolytic killer activity from apoptosis using ImmunoChemistry Technologies' (ICT) Total Cytotoxicity & Apoptosis Detection Kit. It will quantify 4 populations of cells: live; early apoptotic; late apoptotic; and necrotic cells.

Because it can detect cells in early apoptosis, which cannot be detected by any other method, at least 10% more potential cytolytic activity is often measured. 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 Total Cytotoxicity & Apoptosis Detection Kit is the best method to accurately quantify 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[3]. 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[3]. Unfortunately, these techniques have several drawbacks such as: high spontaneous leakage resulting in high backgrounds; high cost; short half-life; the health risks due to exposure to radioactive material[8]; and the inability to detect early-stage apoptotic cells.

Flow cytometric assays have been developed to overcome some of the difficulties associated with older assays like lactate dehydrogenase and ^{51}Cr release assays. Radosevic[7] detected NK cytotoxicity activity by staining target cells with the green fluorescent dye, F-18, in combination with the DNA intercalating dye, propidium iodide[7]. 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[2,4,5,9], 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[3] compared it with the ^{51}Cr release assay and demonstrated a correlation greater than 95%[3].

Since then, the problems with older flow cytometric assays were overcome when Olin[6] used 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) to stain the membranes of target cells[6]. 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[6], ICT has improved the flow cytometric assay by combining it with ICT's SR-FLICA® apoptosis detection reagent to concurrently quantify caspase-positive cells. ICT's Total Cytotoxicity & Apoptosis Detection Kit includes three fluorescent reagents: CFSE; SR-FLICA®; and 7-AAD; which enable the flow cytometer to easily separate the target and effector cell populations for analysis (Figure 1). 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 membrane stain, is used to label all of the target cells green (Figure 4). The unstained effector cells are then added and incubated with the target cells (referred to as the 'E:T' mixture; Figure 5). Apoptotic target cells can then be identified by labeling with the second reagent, SR-FLICA® (Figure 6). SR-FLICA® is an orange/red fluorescent poly caspase inhibitor, SR-VAD-FMK, which binds to active caspase enzymes up-regulated for apoptosis[1]. Upon completion of the E:T incubation (which includes exposure to the apoptosis detection reagent), the last 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 (Figure 7).

8 control populations are used to compensate the flow cytometer (Figures 3, 8-13). As all of the target cells are initially labeled green with CFSE, and the effector cells are not, these two populations can easily be distinguished. Proper gating of the orange/red SR-FLICA® caspase-specific signal in FL-2 and the red 7-AAD live/dead signal in FL-3 distinguishes between apoptotic and necrotic cells within a single sample tube.

Staining with SR-FLICA® is essential to quantify cell death as 7-AAD will not detect cells in the early stages of apoptosis. When used together, this test often reveals a significant percentage of cells were 7-AAD-negative (indicating that they are alive and do not have compromised membranes) but are SR-FLICA®+positive (meaning that they are becoming apoptotic and dying and have active caspase enzymes). These cells cannot be detected by any other method thereby underestimating the true level of cytotoxicity by at least 10% (Figures 15-17). By including all apoptotic events to analyze cell death, ICT's Total Cytotoxicity & Apoptosis Detection Kit is the most accurate method to calculate cytotoxicity.

2. KIT CONTENTS

Catalog #971, trial size, 125 tests, contains:

- 1 vial of CFSE green membrane stain, approximately 250 tests, #6162; store at ≤-20°C.
- 1 vial of SR-VAD-FMK FLICA® orange/red poly caspase inhibitor, approximately 125 tests, #6221; store at 2-8°C or frozen.
- 1 vial of 7-AAD red 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 #972, regular size, 250 tests, contains:

- 1 vial of CFSE green membrane stain, approximately 250 tests, #6162; store at ≤-20°C.
- 2 vials of SR-VAD-FMK FLICA® orange/red poly caspase inhibitor, approximately 125 tests each, #6221; store at 2-8°C or frozen.
- 2 vials of 7-AAD red 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 may be stored frozen, however some of the components may be 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.
- SR-VAD-FMK, 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

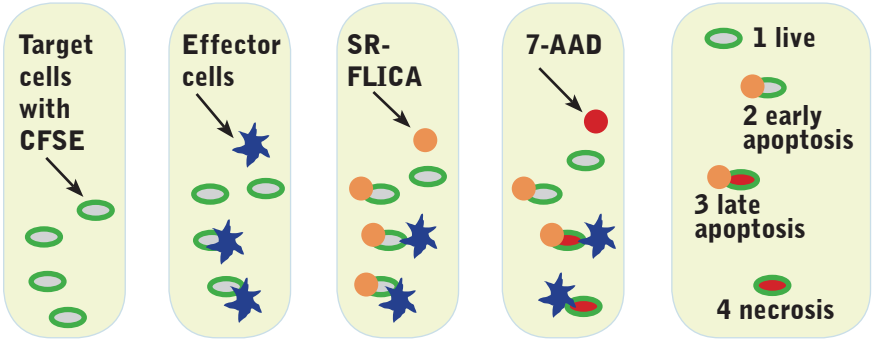
- DMSO, up to 1 mL to reconstitute reagents
- DiH_20, 270-540 mL to dilute Assay Buffer
- Phosphate buffered saline (PBS) pH 7.4, up to 100 mL, to dilute SR-FLICA® and handle cells
- Cultured cells or tissues treated with the experimental conditions ready to be labeled
- Reagents to induce cytotoxicity, apoptosis, and create controls, such as staurosporine (cat. #6212) or camptothecin (cat. #6210)
- 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:

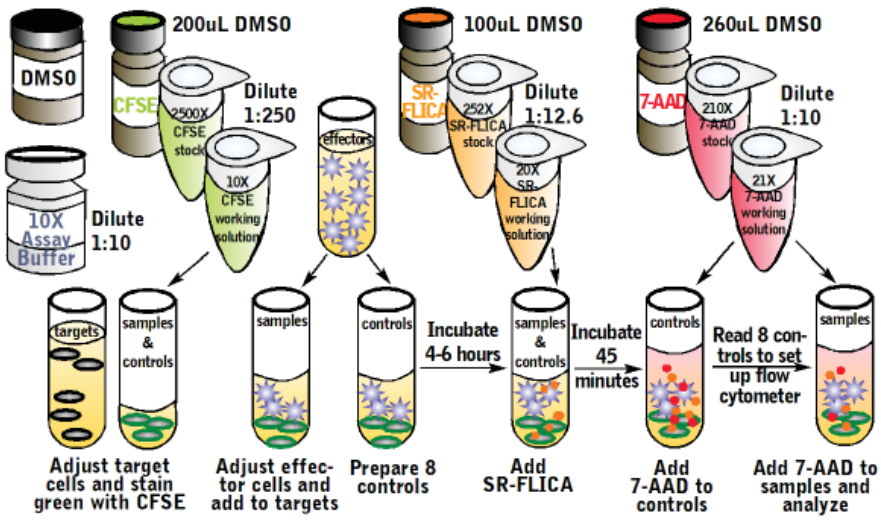
- CFSE green membrane stain: excitation at 492 nm; emission at 520-540 nm in FL-1 (Section 14)
- SR-FLICA® orange/red caspase inhibitor reagent: excitation at 565 nm; emission at 590-600 nm in FL-2 (Section 16)
- 7-AAD red live/dead vital stain: excitation at 546 nm; emission at 647 nm in FL-3 (Section 17)

FIGURE 1: QUANTITATE 4 POPULATIONS IN EACH SAMPLE



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 orange/red SR-FLICA® apoptosis reagent. It will enter the cells and bind to active caspases. If the cells are not apoptotic, it will wash out of the cells.	Add the red live/dead stain, 7-AAD. It will enter cells with compromised membranes in necrosis and late apoptosis and bind to DNA. It will not enter cells with intact membranes.	Run 8 controls and analyze using flow cytometry to quantify 4 populations of target cells in each sample, including cells in early apoptosis which are not detectable by any other method: 1) live target cells; 2) early apoptotic cells; 3) late apoptotic cells; and 4) necrotic cells.

FIGURE 2: ASSAY PROCEDURE



ImmunoChemistry TECHNOLOGIES LLC

7. OVERVIEW

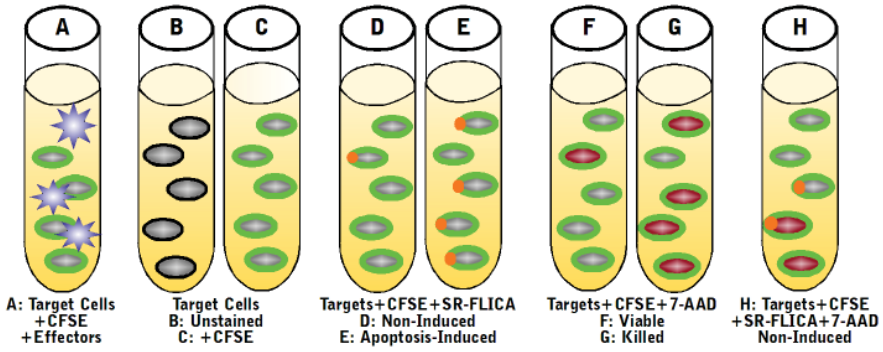
Quantifying cell death with ICT's Total Cytotoxicity & Apoptosis Detection Kit can be completed within a few hours. However, the experiment is performed on living cells, which require periodic maintenance and cultivation several days in advance. Once the proper number of cells has been cultivated, time must be allotted for the experimental treatment, cytotoxic process, or to induce apoptosis. 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, eight control tubes must be prepared for compensation of the flow cytometer (Section 8).

ICT's Total Cytotoxicity & Apoptosis Detection Kit includes 10X Assay Buffer, and three lyophilized fluorescent reagents: CFSE; SR-FLICA®; and 7-AAD which must be reconstituted and diluted prior to use. First dilute and filter the 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.
2. Reconstitute CFSE with 200 µL DMSO.
3. Reconstitute SR-FLICA® with 100 µL DMSO.
4. Reconstitute 7-AAD with 260 µL DMSO.
5. Dilute 2500X CFSE stock 1:250 in sterile 1X Assay Buffer (4 µL into 996 µL).
6. For each sample, prepare a 1.8 mL solution of target cells at 1-2 x 10^6 cells/mL in 1X Assay Buffer (Section 12).
7. Prepare 8 control tubes (Section 8).
8. Add 200 µL diluted 10X CFSE to target cells, and all controls except B (at 1.8 mL).
9. Incubate 15 minutes at RT.
10. Wash cells: add 1 mL media, centrifuge, remove supernatant, add 2-3 mL media.
11. Incubate 30 minutes at 37°C.
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 (forming 200 µL of the 'E:T' mixture) and Control A.
14. Incubate 3.25-5.25 hours at 37°C.
15. Dilute 252X SR-FLICA® stock 1:12.6 in media (10 µL into 116 µL).
16. Add 10 µL diluted 20X SR-FLICA® to E:T, Controls D, E, & H (at 200 µL).
17. Incubate 45 minutes at 37°C; wash cells if desired.
18. Add 200 µL RPMI and place cells on ice (total of 400 µL).
19. Kill cells (Section 10) to create Control G.
20. Dilute 210X 7-AAD stock 1:10 in sterile 1X Assay Buffer (40 µL into 360 µL).
21. Add 20 µL diluted 21X 7-AAD to Controls F, G, & H.
22. Incubate controls 10 minutes on ice.
23. Run the 8 instrument controls to set up the proper gates and adjust compensation (Section 8, Figures 8-13).
24. Add 20 µL diluted 21X 7-AAD to samples (at 400 µL).
25. Incubate samples 10 minutes on ice.
26. Read and analyze samples (Section 20).

FIGURE 3: 8 INSTRUMENT CONTROLS



8. FLOW CYTOMETRY CONTROLS

8 control tubes (Figure 3) are needed to properly gate the flow cytometer and set up compensation to obtain the most accurate results (Section 20). Follow Figures 8-13 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 8. It is also used as a negative control in Figure 12.

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 9.

D&E: D and E contain target cells labeled green with CFSE and orange/red SR-FLICA®. The cells in D are not induced to undergo apoptosis, while the cells in E are induced (Section 9). They determine the shift of SR-FLICA® from left to right in Figure 10.

F&G: F and G contain target cells stained green with CFSE and red with 7-AAD. F contains live cells, while the cells in G are killed (Section 10). They will determine the shift of 7-AAD from bottom to top in Figure 11.

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

9. APOPTOSIS INDUCTION

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

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10. METHODS TO KILL CELLS

In Section 8, Control G (Figure 11) 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, shifting the population to the left.

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

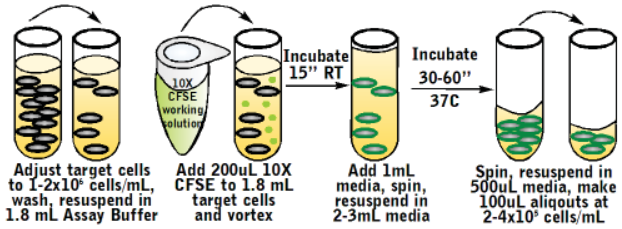


FIGURE 5: ADD EFFECTOR CELLS

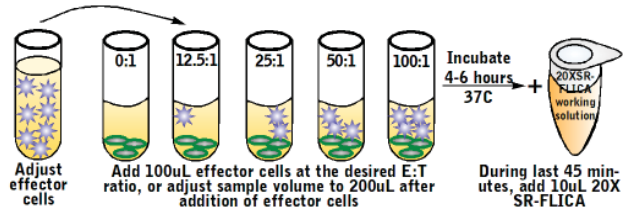


FIGURE 6: LABEL EARLY AND LATE APOPTOTIC CELLS WITH SR-FLICA®

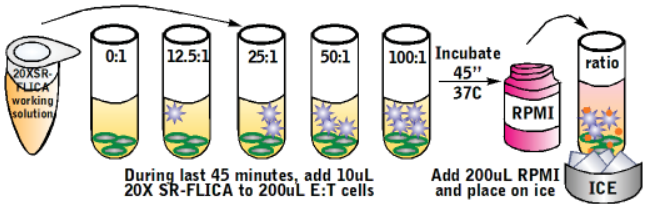
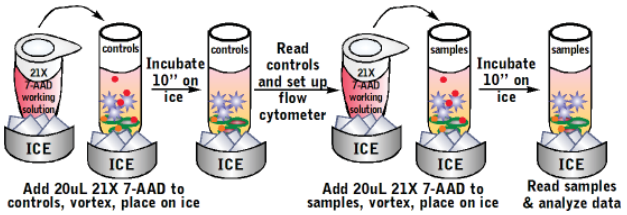


FIGURE 7: LABEL NECROTIC CELLS WITH 7-AAD



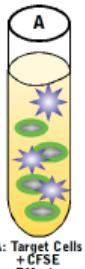
PROTOCOL

11. PREPARE SAMPLES AND CONTROLS

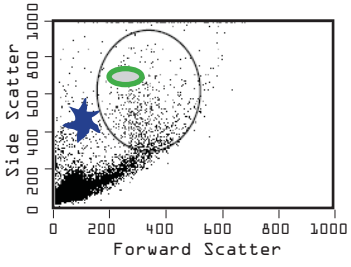
All target cells must be stained green with CFSE to distinguish them from non-stained effector cells (Figure 4). If studying the effects of a toxic agent rather than cells, CFSE staining is optional as the only cells present will be the target cells.

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.

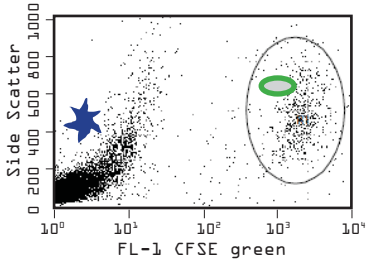
FIGURE 8: CONTROL A WILL DISTINGUISH GREEN CFSE-LABELED TARGET CELLS FROM UNSTAINED EFFECTOR CELLS: FSC VS. SSC AND FL-1 (CFSE) VS. SSC PLOTS



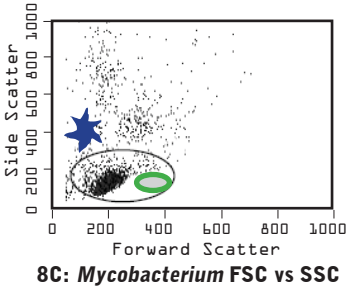
Control A contains CFSE-stained target cells and effector cells (E:T). It is used to distinguish the green CFSE-stained target cells from the unstained effector cells. Run Control A and create a forward scatter (FSC) vs. side scatter (SSC) plot (Figures 8A and 8C). Large cells, like K562 cells (circled)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



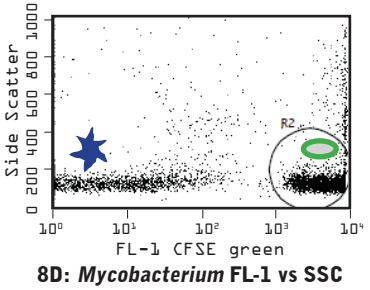
8A: Control A FSC vs SSC



8B: Control A FL-1 vs SSC



8C: *Mycobacterium* FSC vs SSC



8D: *Mycobacterium* FL-1 vs SSC

12. 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 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 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 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.

13. ADJUST TARGET CELLS IN ASSAY BUFFER

5. Adjust target cells to 1-2 x 10^6 in 1 mL 1X Assay Buffer. 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 2-3mL 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.

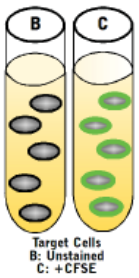
14. STAIN TARGET CELLS WITH CFSE

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.

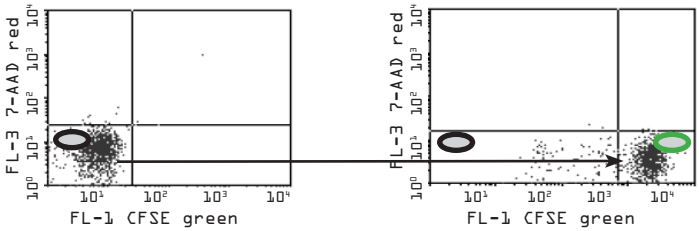
CFSE must first be reconstituted in DMSO, forming a 2500X stock concentrate, and then diluted 1:250 in sterile 1X Assay Buffer to form the 10X working solution that will be used to stain the target cells. Do NOT dilute CFSE in media, as the cell membrane reactive properties of the CFSE stain will be 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.

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



Control B contains unstained target cells. Control C contains CFSE-stained target cells. Run Control B containing unstained target cells (Figure 9A) and then run Control C containing CFSE-stained target cells (Figure 9B) 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 Control B. Adjust the PMT voltage so that the stained target cells fall within the 3rd or 4th decade and save the data to ensure the target cells are properly gated {data 072204}.

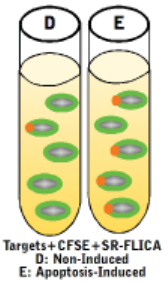


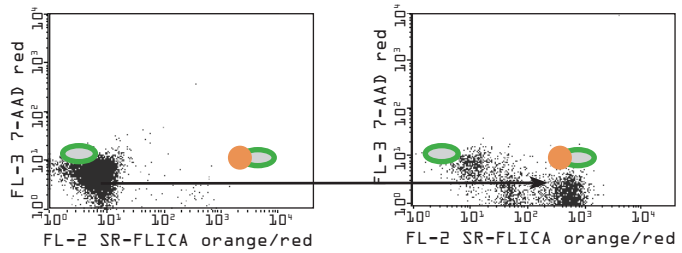
9A: Control B Unstained Target Cells

9B: Control C CFSE-Labeled Green Target Cells

FIGURE 10: CONTROLS D&E DISTINGUISH NON-APOPTOTIC FROM APOPTOTIC TARGET CELLS: FL-2 VS. FL-3

Control D contains target cells labeled with CFSE and SR-FLICA that were not induced to undergo apoptosis (it is the negative control). Control E contains target cells labeled with CFSE and SR-FLICA® that were induced to undergo apoptosis. Run Control D to properly compensate FL-2 SR-FLICA® vs. FL3 7-AAD (Figure 10A). Depending upon each cell line, a small percentage of the non-induced control population will naturally undergo apoptosis and appear to the right (Figure 10A). Run Control E (Figure 10B). Compared with non-induced cells (Control D, Figure 10A), apoptotic cells will shift to the right (Figure 10B; not all cells will undergo apoptosis). These cells have not been stained with 7-AAD; if they were, compensation could not be determined, as dead cells would migrate up the plot (Figure 11B), obscuring the data. Apoptotic target cells (Control E) exhibit high levels of staining with SR-FLICA® and shift to the right compared to non-apoptotic cells (Control D) {data 021705}.





10A: Control D Non-Induced Target Cells

10B: Control E Apoptotic Target Cells

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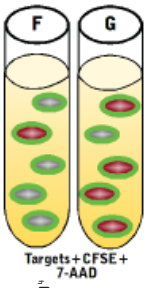
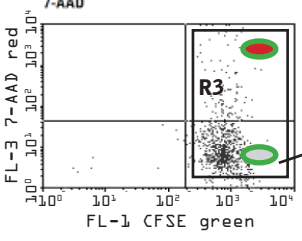
12. When ready to use in the assay, dilute the 2500X stock 1:250 with sterile 1X Assay Buffer. For example, add 4 µL of 2500X CFSE stock to 0.996 mL sterile assay buffer and mix. This yields 1 mL of 10X CFSE working solution. For best results, the 10X working solution should be used within 2 hours, stored on ice, and protected from light. Do NOT dilute in media, as the fluorescence will be quenched.
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 (or other condidtions appropriate for the experiment). 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 cells with 500 µL media.
21. Adjust the concentration of the target cells using cell culture media to 2-4 x 10^5 cells/mL or 2-4 x 10^4 in 100 µL. We recommend starting with this concentration, as too many cells will induce spontaneous cell death.

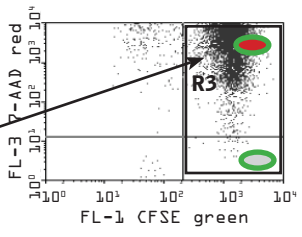
FIGURE 11: CONTROLS F&G WILL DISTINGUISH LIVING FROM DEAD TARGET CELLS: FL-1 VS. FL-3

Control F contains live target cells labeled with CFSE and 7-AAD. Control G contains killed target cells stained with CFSE and 7-AAD. Run Control F to compensate FL-1 (CFSE) vs. FL-3 (7-AAD, Figure 11A). These cells must be viable: compare with the killed cells (Control G, Figure 11B) 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 the cell line. Run Control G containing killed CFSE-stained target cells dually stained with 7-AAD (Figure 11B). Killed target cells stained green with CFSE & red with 7-AAD (Control G) migrate up the plot compared to live cells (Control F). Create a gate (R3, in Figure 11A) and rerun Control A (Figure 12). See Section 10 for 2 methods to kill cells {data 072204}.





11A Control F, live target cells



11B Control G, dead target cells

15. ADD EFFECTOR CELLS

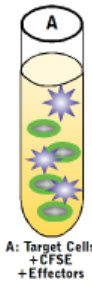
22. Adjust the concentration of the effector cells so that approximately 100 µL can be added to the target cells yielding the desired Effector:Target cell ratio (E:T ratio), such as 50:1 (Figure 5). For example, if 100 µL target cells are at 1.5 x 10^4 (Section 15), add 50 times that amount of effector cells (75 x 10^4) in 100 µL. The concentration of effector cells would have to be 750 x 10^4 = 7.5 x 10^6 cells/mL. An optimal E:T cell ratio is required to effectively determine cytolytic activity.
23. Add effector cells to samples and Control A and adjust the volume to 200 µL.
24. Incubate 4-6 hours. The incubation time may vary depending on the experiment.
25. Approximately 1 hour prior to the end of the incubation period, prepare SR-FLICA® and add it (Section 16).

16. LABEL APOPTOTIC CELLS WITH SR-FLICA®

Cells that are in the early stages of apoptosis can be detected by ICT's Fluorochrome Labeled Inhibitors of CAspases reagents (FLICA®). ICT's orange-red poly caspase SR-FLICA® inhibitor reagent, sulforhodamine-labeled Valine-Alanine-Aspartic-acid fluoromethyl ketone (SR-VAD-FMK, catalog #6221), has been optimized for this kit. Just add the inhibitor to the mixture of target and effector cells, and caspase-positive cells will fluoresce orange-red.

FIGURE 12: RERUN CONTROL A: FL-2 VS. FL-3

Control A contains CFSE-stained target cells and effector cells (E:T). It is also used in Figure 8. Using the compensation set up for Control F, draw a gate around the CFSE and 7-AAD positive target cells (R3 in Figure 11A). Set acquisition to collect events within R3. Create a dot plot of FL-2 SR-FLICA® vs. FL-3 7-AAD gated off R3. Rerun Control A under new conditions, FL-2 vs. FL-3, as a negative control to ensure proper compensation of the machine (Figure 12). Once gating is set (Figure 11A), CFSE-stained target cells appear in the lower left quadrant of this plot (Figure 12). Since this control has not been stained with either of the red labels, (7-AAD nor SR-FLICA®) there should be very few cells in the background {data 012706}.



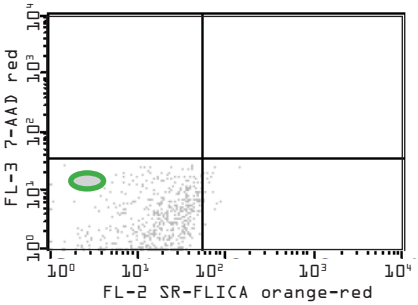
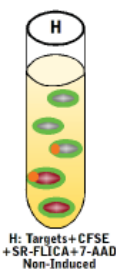
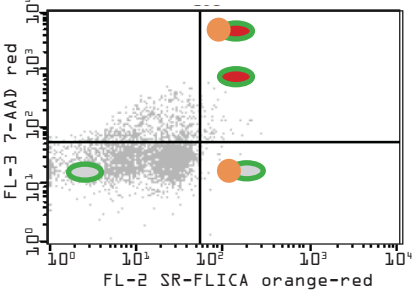


FIGURE 13: CONTROL H, FL-2 VS. FL-3

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





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SR-FLICA® is cell-membrane-permeant, so there is no need to lyse the cells; it will enter the cell and form a covalent bond with active caspase enzymes inside the cell undergoing apoptosis. Because SR-FLICA® is always fluorescent, it may be necessary to remove any unbound SR-FLICA® from the cells or media by washing the cells.

It is important to expose the target cells to SR-FLICA® as 7-AAD may not detect cells in the early stages of apoptosis. Careful gating of SR-FLICA® and 7-AAD fluorophores (using the FL-2 and FL-3 channels respectively), distinguishes the orange-red SR-FLICA® caspase-specific signal from the red 7-AAD live/dead fluorescence signal within a single sample tube. This assay will often reveal a significant percentage of early apoptotic cells that were 7-AAD negative (indicating they are alive) yet SR-FLICA®-caspase positive (indicating they are entering apoptosis). These early-apoptotic-soon-to-be-necrotic cells can then be included in the overall percentage of total cell death leading to more accurate results.

The SR-FLICA® inhibitor reagent is supplied as a lyophilized powder that may be slightly visible as an iridescent sheen inside the vial. The amber vial may appear empty; once reconstituted in DMSO, it may remain colorless or slightly colored. It must first be reconstituted in DMSO, forming a 252X stock concentrate, and then diluted 1:12.6 in sterile Assay Buffer (or media) to form a final 20X working solution that will be used to label the cells. Store lyophilized SR-FLICA® at 2-8°C or ≤-20°C protected from light.

26. Reconstitute SR-FLICA® with 100 µL DMSO. This yields a 252X 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, the reagent should be dissolved within a few minutes. Store the 252X stock concentrate at ≤-20°C protected from light.
27. If not using all the 252X stock at the time it is reconstituted, store it at ≤-20°C for 6 months protected from light. To avoid freeze-thaw cycles, make small aliquots in amber vials or polypropylene tubes.
28. When ready to use in the assay, dilute the 252X stock 1:12.6 with sterile 1X Assay Buffer or media and mix. For example, add 10 µL of the 252X stock to 116 µL 1X Assay Buffer. This yields 126 µL of the 20X working solution. For best results, the 20X working solution should be used within 4 hours, stored on ice, and protected from light.
29. Add 10 µL 20X SR-FLICA® working solution to controls D, E, and H and sample tubes (at 200 µL) and mix.
30. Incubate for the remaining 45 minutes of E:T incubation at 37°C protected from light.
31. An optional wash step may be necessary to remove any unbound SR-FLICA® from the media: centrifuge <300 x g for 5 minutes, discard supernatant, and resuspend in 200 µL RPMI.
32. Add 200 µL RPMI for a total volume of 400 µL and place on ice.

FIGURE 14: IDENTIFY GREEN TARGET CELLS

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

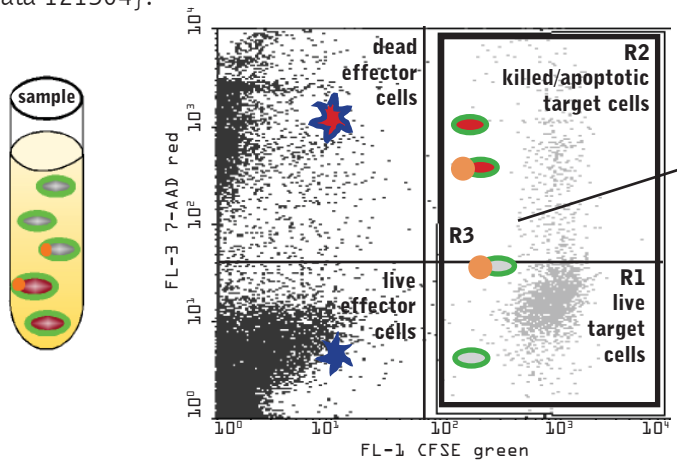


FIGURE 15: QUANTITATE 4 POPULATIONS

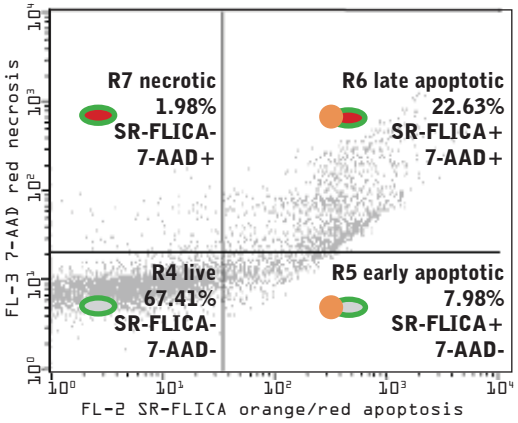
Once the green target cells have been gated (R3, Figure 14), derive a plot of the poly caspase apoptosis reagent (SR-FLICA®) in FL-2 vs. live/dead viability stain (7-AAD) in FL-3. This plot reveals 4 populations of cells: live cells and 3 populations of cells in the death process, including cells in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

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

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

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

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



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17. LABEL CONTROLS WITH 7-AAD

Dead target cells can be identified with 7-aminoactinomycin D (7-AAD, catalog #6163). 7-AAD is a red vital stain that can be used to identify and quantitate dead and dying target cells resulting from the cytolytic activity of the effector cells or toxic agent. This dye will penetrate the structurally 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 (Figure 7). Because 7-AAD may not stain cells in the early stages of apoptosis, SR-FLICA® is needed to concurrently detect apoptotic cells.

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.

33. Reconstitute lophilized 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.
34. 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.
35. 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.
36. Add 20 µL 21X 7-AAD to Controls F, G, and H (at 400 µL) just prior to analysis and mix or gently vortex. Do not add 7-AAD to the experimental samples until the flow cytometer has been compensated.
37. Incubate 10 minutes on ice protected from light.

FIGURE 16: ACCURATE CALCULATION OF CYTOTOXICITY

Based on the percentage of cells in each quadrant total cytotoxicity can be calculated accurately: it is 32.59%. This analysis reveals a population in the lower right quadrant of early apoptotic cells that is not detectable by PI staining or any other method (7.98% are SR-FLICA® positive, but 7-AAD negative). These early-apoptotic soon-to-be-necrotic cells can then be included in the overall percentage of total cell death leading to more accurate results. By including all apoptotic events to analyze cytotoxicity (7.98% + 22.63% + 1.98%), cytotoxicity can now be accurately quantitated as 32.59%. Other assays would calculate cytotoxicity at only 24.61% because they miss the 7.98% of cells that are in early apoptosis, thereby underestimating the true level of cytotoxicity by 25%! ICT's Total Cytotoxicity & Apoptosis Detection Kit is the best assay to accurately quantify cytotoxicity because it includes cells in early apoptosis {data 101805}.

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

18. RUN CONTROLS AND SET UP FLOW CYTOMETER

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

19. LABEL SAMPLES WITH 7-AAD

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

20. ANALYZE SAMPLES

41. Distinguish green target cells from unstained effector cells by running FSC vs. SSC or FL-1 vs. SSC. Gate on the target cell population.
42. Create a plot of CFSE (FL-1) vs. 7-AAD (FL-3) and gate on the green CFSE-stained target cells (R3 in Figure 14).
43. From this population of green target cells, prepare a dot plot of SR-FLICA® (FL-2) vs. 7-AAD (FL-3) (Figure 15).
44. Calculate cytotoxicity (Figures 16 and 17).

21. REFERENCES & CITATIONS

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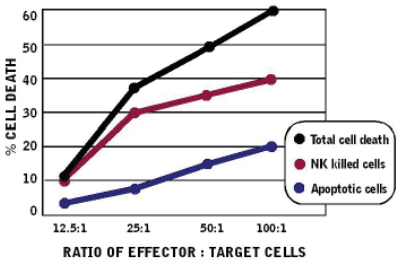
FIGURE 17: RATIO OF CYTOLYTIC ACTIVITY

K562 target cells were stained with CFSE and adjusted to 1.5 x 10^4. Effector cells were added at a ratio of 0:1, 12.5:1, 25:1, 50:1, or 100:1. Cells were incubated for 4 hours to allow the cytolytic activity to progress. Cells were labeled with SR-FLICA® and 7-AAD and analyzed via flow cytometry following Figures 14, 15, and 16. Total cytotoxicity increases as more effector cells are combined with target cells. Using just 7-AAD, NK-killed cells are revealed as R6+R7 (maroon line). By including SR-FLICA®, apoptotic cells are also revealed (R5, blue line). When combined, the total % of cell death is calculated (black line) versus the effector:target cell ratio. CTL activity increased when more effector cells were used. The true level of cytotoxicity is revealed by including apoptotic cells (20% of cells were apoptotic at 100:1) which would have been undetected using other methods.



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