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ab270781 Apoptosis/ Necrosis Assay Kit (green, orange, red)

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For the measurement of cytolytic activity in cultured cells.

This product is for research use only and is not intended for diagnostic use.

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1. Overview

Cytolytic activity is an important process for eliminating intracellular pathogens and cancer cells. Apoptosis/ Necrosis Assay Kit (green, orange, red) (ab270781) allows you to assess cytolytic activity in cell culture.

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. Unfortunately, these techniques have several drawbacks. Traditional enzyme-release assays are often skewed by the large number of necrotic effector cells. Problems associated with ^{51}Cr release methods include high spontaneous leakage resulting in high backgrounds, high cost, short half-life, and the health risks due to exposure to radioactive material. Beyond these limitations, these assays frequently underestimate the true level of cytotoxicity, as they are unable to detect early-stage apoptotic cells.

Flow cytometric assays have been developed to overcome some of the difficulties associated with older assays like lactate dehydrogenase and ^{51}Cr release assays. One such early version involved the detection of NK cytotoxicity activity by staining target cells with the green fluorescent dye, F-18, in combination with the DNA intercalating dye, propidium iodide . 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 iodide4-7. However, despite correlations of greater than 95% when compared with the ^{51}Cr release assay, the PKH-26 method is problematic. It is difficult to use at a constant concentration, leading to unreliable staining, and the staining procedure requires multiple steps, often decreasing the viability of the target cells.

Apoptosis/ Necrosis Assay Kit (green, orange, red) is a flow cytometric assay combining the green fluorescing cellular stain, (CFSE), with a red fluorescing live/dead stain, 7-aminoactinomycin D (7-AAD), and SR-VAD-FMK apoptosis detection reagent to concurrently quantify caspase-positive cells. The assay can be used to determine total cytotoxicity in the form of apoptosis and necrosis. It will quantify 4 populations of cells: live; early apoptotic; late apoptotic; and necrotic cells within a single sample tube.

2. Materials Supplied and Storage

Store kit at -20°C immediately on receipt. However, some components may be stored at +4°C.

Δ Note: Once reconstituted with DMSO, use CFSE, SR-VAD-FMK and 7-AAD immediately, or store at ≤-20°C for 6 months protected from light and thawed no more than twice during that time.

Δ Note: 10X Assay Buffer may be stored frozen or refrigerated. 1X Assay Buffer may be stored at 4°C for 1 week, or frozen and used within 6 months..

Item	Quantity		Storage temperature
	125 tests	250 tests	
CFSE, green cellular stain, 250 tests	1 vial	1 vial	-20°C
Caspase inhibitor orange red SR-VAD-FMK	1 vial	2 vials	+4°C or -20°C
10X Assay Buffer	1 x 30 mL	1 x 60 mL	+4°C or -20°C
7-AAD, red live/dead stain, 125 tests	2 vials	2 vials	+4°C or -20°C

3. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- DMSO, up to 1 mL to reconstitute reagents
- DiH_2O, 270-540 mL to dilute 10X Assay Buffer
- Phosphate buffered saline (PBS) pH 7.4, 100 mL
- Cultured cells treated with the experimental conditions ready for labelling
- Reagents to induce apoptosis and create controls, such as staurosporine or camptothecin
- 15% ETOH (in PBS or 1X Assay Buffer) to create live/dead controls for 7-AAD staining
- Hot water bath, to create live/dead controls for 7-AAD staining
- Centrifuge at 200 *x g*
- Hemocytometer
- 15 mL polypropylene centrifuge tubes (1 per sample)
- FACS tubes
- Flow cytometer
- Ice bath
- 37°C incubator

4. General guidelines, precautions, and troubleshooting

Please observe safe laboratory practice and consult the safety datasheet.

For general guidelines, precautions, limitations on the use of our assay kits and general assay troubleshooting tips, particularly for first time users, please consult our guide:
www.abcam.com/assaykitguidelines

For typical data produced using the assay, please see the assay kit datasheet on our website.

5. Experimental Overview

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

Control populations must be made for the experimental conditions. If the experiment is designed to determine innate or adaptive cytotoxicity using isolated macrophages or monocytes, etc., prepare a control of non-infected target cells combined with effector cells to determine cell death which normally occurs within the healthy target cells. In addition, several control tubes must be prepared for compensation and gating of the flow cytometer (Section 6).

The kit includes 10X Assay Buffer, and three lyophilized fluorescent reagents: CFSE, SR-VAD-FMK, and 7-AAD, which must be reconstituted and diluted prior to use. First dilute and filter the 10X Assay Buffer, as it is used to dilute the other reagents. Then reconstitute the lyophilized reagents with DMSO to create the stock concentrates and store on ice. Once it is time to use the reagent, prepare the working solution by diluting the stock. Here is a quick overview of the procedure.

5.1 Dilute 10X Assay Buffer 1/10 with diH_20, forming 1X Assay Buffer and sterile filter (Section 9).

5.2 Reconstitute CFSE with 200 µL DMSO, forming a 2500X stock concentrate (Section 12).

5.3 Dilute 2500X CFSE stock 1/250 in sterile 1X Assay Buffer, forming a 10X working solution (4 µL into 996 µL).

5.4 For each sample and control, adjust target cells to 1-2 x 10^6 cells/mL, wash, and resuspend each in 1.8 mL 1X Assay Buffer (Section 11).

5.5 Prepare control tubes (Section 6).

5.6 Add 200 µL diluted 10X CFSE to target cells, and all controls except B (at 1.8 mL).
5.7 Incubate 15 mins at room temperature.
5.8 Wash cells: add 1 mL media, centrifuge, remove supernatant, and add 2-3 mL media.
5.9 Incubate 30 mins at 37°C.
5.10 Adjust stained target cells to 2 x 10^5 cells/mL and make 100 µL aliquots (2 x 10^4 cells/aliquot).
5.11 Adjust unstained effector cells to the desired concentration, such as 50 times the concentration of the target cells, in 100 µL aliquots.
5.12 Add 100 µL unstained effector cells to the CFSE stained target cells and Control A. This forms the 'E:T' mixture at 200 µL.
5.13 Incubate 'E:T' mixture 4-6 hours at 37°C.
5.14 Create a positive control of apoptotic cells to generate Control E (Section 7).
5.15 Reconstitute SR-VAD-FMK with 100 µL DMSO, forming a 252X stock concentrate (Section 16).
5.16 Dilute 252X SR-VAD-FMK stock 1/12.6 in media, forming a 20X working solution (10 µL into 116 µL).
5.17 Add 10 µL diluted 20X SR-VAD-FMK to 'E:T' experimental samples, and Controls D, E, and H (at 200 µL).
5.18 Incubate 45 mins at 37°C. Wash cells and resuspend in 400 µL media; place cells on ice.
5.19 Create a positive control of killed cells to generate Control G (Section 9).
5.20 Reconstitute 7-AAD with 260 µL DMSO, forming a 210X stock concentrate (Section 15).
5.21 Dilute 210X 7-AAD stock 1:10 in sterile 1X Assay Buffer, forming a 21X working solution (40 µL into 360 µL).
5.22 Add 20 µL diluted 21X 7-AAD to Controls F, G, and H (at 400 µL).
5.23 Incubate controls 10 mins on ice.
5.24 Run the instrument controls to set up gating and compensation on the flow cytometer (Section 8).
5.25 Add 20 µL diluted 21X 7-AAD to samples (at 400 µL).
5.26 Incubate samples 10 mins on ice.
5.27 Read and analyze samples (Section 18).

6. Flow Cytometry Controls

Several control populations are needed to properly gate the flow cytometer and set up compensation on the instrument (Section 16). Follow the table below to create the control tubes and set up the flow cytometer. Examples shown here were generated on a BD FACS Caliber; compensation requirements may differ among instruments.

Control #	
A	Control A contains target cells stained with CFSE and effector cells. It is used to separate the green target cells from the unstained effector cells. It is also used as a negative control to ensure proper gating of SR-VAD-FMK and 7-AAD reagents
B and C	Control B contains unstained target cells. Control C contains target cells stained green with CFSE. They are used to determine the shift of target cells from left to right along FL-1
D and E	Control D contains healthy target cells stained green with CFSE and orange/red with SR-VAD-FMK (non-induced negative control). Control E contains apoptotic target cells stained green with CFSE and orange/ red with SR-VAD-FMK (induced to undergo apoptosis) (Section 7). They determine the shift of SR-VAD-FMK from left to right along FL-2
F and G	Control F contains live target cells stained green with CFSE and red with 7-AAD. Control G contains killed target cells stained green with CFSE and red with 7-AAD (Section 9). They will determine the shift of 7-AAD from bottom to top along FL-3
H	Control H contains target cells stained with CFSE, SR-VAD-FMK, and 7-AAD that are not induced to undergo apoptosis. It will determine background levels of apoptosis and necrosis without the influence of effector cells.

7. Apoptosis Induction

In Section 6, Control E is created as an apoptosis-positive control to verify staining with SR-VAD-FMK. 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 or 1-2 µM staurosporine for >4 hours.

8. Preparation of Samples and Controls

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

Cultivate the proper number of target and effector cells for the sample and control populations. Allow time for the experimental treatment or cytotoxic process. Do not use target cells that are capable of proliferating more than 4 hours prior to the assay; when a CFSE labeled cell divides, its progeny each inherit half the number of fluorescent tagged molecules as the parent cell. Therefore, proliferation will decrease the average fluorescence intensity of the target cell population.

Δ Note: As cell media will quench CFSE fluorescence, the media must be replaced with 1X Assay Buffer before staining with CFSE.

9. Prepare Killed Cells

In Section 6, Control G contains target cells labeled green with CFSE that have been killed and then labeled red with 7-AAD. For compensation and gating, it is preferable to use a control that contains a mixture of live and dead cells. Below are 2 methods for preparing such a control.

Method 1: Hot water bath.

- Immerse the tube of cells in a 56°C water bath for 10-20 mins. The optimal heat exposure period may vary with cell type. For best results, determine a reproducible method for killing 30-60% of the cell population prior to commencing the experiment.
- Place on ice.
- Add 7-AAD to stain necrotic cells (Section 15).

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

- Centrifuge cells at 200 x *g* for 5-10 mins at room temperature (RT).
- Carefully remove the supernatant.
- Resuspend cells in 15% ethanol. For example, add 150 µL ethanol plus 850 µL PBS or 1X Assay Buffer to resuspend cells.
- Incubate 10 mins at RT.
- Add 2-3 mL 1X Assay Buffer.
- Centrifuge at 200 x g for 5-10 mins at RT.
- Carefully remove the supernatant.
- Add 400 µL media to resuspend cells.
- Add 7-AAD to stain necrotic cells (Section 15).

10. Preparation of 1X Assay Buffer

10X Assay Buffer 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 ≤4°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 4°C for 1 week or frozen and used within 6 months.

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

- 10X Assay Buffer may form precipitates during cold storage. If this happens, gently warm it until all crystals have dissolved. Do not boil.
- 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).
- Mix for 5 mins or until all crystals have dissolved.
- Sterilize by filtration.

11. Adjust Target Cells in 1X Assay Buffer

11.1 Adjust target cells to 1-2 x 10^6 cells/mL in 1 mL 1X Assay Buffer Do not use media as it will quench CFSE fluorescence.

11.2 Wash target cells twice with 1X Assay Buffer to remove any media. Centrifuge at 200 x *g* for 5-10 mins at RT and discard supernatant.

11.3 Resuspend target cells with 2-3 mL 1X Assay Buffer.

11.4 Centrifuge at 200 x *g* for 5-10 mins at RT; discard supernatant.

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

12. Stain Target cells with CFSE

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

CFSE must first be reconstituted in DMSO, forming a 2500X stock concentrate, and then diluted 1/250 in sterile 1X Assay Buffer to form the 10X working solution that will be used to stain the target cells.

Δ Note: Do NOT dilute CFSE in media, as the reactive properties of the CFSE stain will be neutralized. Store the lyophilized CFSE and 2500X stock at ≤ -20°C protected from light

12.1 Reconstitute CFSE with 200 µL DMSO. This yields a 2500X stock concentrate. Mix by swirling or tilting the vial, or gently vortexing, allowing the DMSO to travel around the base of the vial until completely dissolved. At room temperature, this should take just a few mins. Protect from light.

12.2 If not using all of the 2500X stock concentrate at the time it is reconstituted, store it at ≤-20°C for 6 months protected from light. To avoid repeated freeze-thaw cycles, make small aliquots in amber vials or polypropylene tubes

12.3 When ready to use in the assay, dilute the 2500X stock 1:250 with sterile 1X Assay Buffer. For example, add 4 µL of 2500X CFSE stock to 996 µL sterile 1X Assay Buffer and mix. This yields 1 mL of 10X CFSE working solution. For best results, the 10X working solution should be used within 2 hours, stored on ice and protected from light.
Δ Note: Do NOT dilute in media, as the fluorescence will be quenched.

12.4 Add 200 µL 10X CFSE working solution to each 1.8 mL suspension of target cells, and to all control tubes except B. Gently vortex.
The optimal concentration of CFSE may vary among cell types. Adjust the concentration of CFSE and the incubation

time to adequately stain the target cells for the experiment. Excessive staining may cause problems when compensating the flow cytometer.

12.5 Incubate 15 mins at room temperature.

12.6 Add 1 mL cell culture media per tube to stop the CFSE binding reaction.

12.7 Centrifuge at 200 x *g* for 5-10 mins at RT; discard supernatant.

12.8 Resuspend in 2-3 mL cell culture media per tube.

12.9 Incubate 30-60 mins at 37°C in a CO_2 incubator (or other conditions appropriate for the experiment). Plan the experiment so the cells incubate no more than 1 hour while setting up the assay.

12.10 Centrifuge at 200 x *g* for 5-10 mins at RT; discard supernatant.

12.11 Resuspend with 500 µL cell culture media. Adjust the concentration of the target cells to 1 x 10^5 cells/mL, therefore a 200 µL volume will contain 2 x 10^4 target cells. The 200 µL aliquots of target cells will be combined with 200 µL of effector cells, yielding the desired Effector: Target cell ratio (E:T), such as 50:1 (Section 10).

13. Add Effector Cells

13.1 Adjust the concentration of the effector cells so that approximately 100 µL can be added to the target cells yielding the desired E:T ratio, such as 50:1.
For example, if the 100 µL target cell suspension contains 2 x 10^4 cells (Section 12), in order to add 50 times that number of effector cells (50 x (2 x 10^4 target cells) = 1 x 10^6 effector cells) in 100 µL, the concentration of effector cells needed would be 1 x 10^7 cells/mL (1 x 10^6 cells ÷ 0.1 mL = 1 x 10^7 cells/mL). An optimal E:T cell ratio is required to effectively determine cytolytic activity.

13.2 Add effector cells to samples and Control A and adjust the total volume to 200 µL.

13.3 Incubate 4-6 hours at 37°C in a CO_2 incubator. The incubation time may vary depending on the experiment.

13.4 Approximately 1 hour prior to the end of the incubation period, prepare SR-VAD-MK and add it to samples and Controls D, E, and H (Section 14).

14. Label Controls with SR-VAD-FMK

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

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

The SR-VAD-FMK 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 appear red in color. It must first be reconstituted in DMSO, forming a 252X stock concentrate, and then diluted 1/12.6 in sterile 1X Assay Buffer (or media) to form a final 20X working solution that will be used to label the cells. Store the lyophilized SR-VAD-FMK refrigerated or frozen; store 252X stock at ≤-20°C protected from light.

14.1 Reconstitute SR-VAD-FMK with 100 µL DMSO. This yields a 252X stock concentrate. Mix by swirling or tilting the vial, or gently vortexing, allowing the DMSO to travel around the base of the amber vial until completely dissolved. Store the 252X stock concentrate at ≤-20°C protected from light.

14.2 If not using all the 252X stock at the time it is reconstituted, store it at ≤-20°C for 6 months protected from light. To avoid repeated freeze-thaw cycles, make small aliquots in amber vials or polypropylene tubes.

14.3 When ready to use in the assay, dilute the 252X stock 1:12.6 with sterile 1X Assay Buffer or media and mix. For example, add 10 µL of the 252X stock to 116 µL 1X Assay Buffer. This yields 126 µL of the 20X working solution. For best results, the 20X working solution should be used within 4 hours, stored on ice, and protected from light.

14.4 Add 10 µL 20X SR-VAD-FMK working solution to Controls D, E, and H and sample tubes (at 200 µL) and mix.

14.5 Incubate for the remaining 45 mins of E:T incubation at 37°C in a CO_2 incubator protected from light.

14.6 Wash cells to remove any unbound SR-VAD-FMK from the media: centrifuge at 200 *x g* for 5-10 mins at RT, discard supernatant, and resuspend in 400 µL media. Place samples on ice.

15. Label Controls with 7-AAD

Dead target cells can be identified with 7-aminoactinomycin D (7-AAD). 7-AAD is a red vital stain that can be used to identify and quantitate dead and dying target cells resulting from the cytolytic activity of the effector cells or toxic agent. This dye will penetrate the structurally compromised cell membranes of dead and dying cells and complex with DNA. The intercalated 7-AAD dye exhibits a red fluorescence in the FL-3 region with maximum output at 647 nm. Staining with 7-AAD should be done just prior to analysis; it is the last step due to its toxic effect on most cell types.

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

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

15.1 Reconstitute lyophilized 7-AAD with 260 µL DMSO. This yields a 210X stock concentrate. Mix by swirling or tilting the vial, or gently vortexing, allowing the DMSO to travel around the base of the amber vial until completely dissolved. At room temperature, it should be dissolved within a few mins.

15.2 If not all of the 210X 7-AAD stock concentrate will be used at the time it is reconstituted, store it at ≤-20°C for 6 months protected from light. To avoid repeated freeze-thaw cycles, make small aliquots in amber vials or polypropylene tubes.

15.3 When ready to use in the assay, dilute the 210X stock 1/10 with sterile 1X Assay Buffer or media. For example, add 40 µL of the 210X stock to 360 µL 1X Assay Buffer and mix. This yields 400 µL of the 21X working solution. For best results, the 21X working solution should be used within 2 hours, stored on ice and protected from light.
Add 20 µL 21X 7-AAD to Controls F, G, and H (at 400 µL) just prior to analysis and mix or gently vortex.

Δ **Note:** Do not add 7-AAD to the experimental samples until the flow cytometer has been set up.

15.4 Incubate 10 mins 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 6).

16.1 Run Control A to separate live, green fluorescing target cells from unstained effector cells. Control A also serves as a negative control on SR-FLICA (FL-2) vs. 7-AAD (FL-3) dot plots. Run Controls B and C to ensure live, green fluorescing target cells fall in the proper region.

16.2 Run Controls B and C to ensure live, green fluorescing target cells fall in the proper region. Run Control F if the experiment is designed to determine innate or adaptive cytotoxicity using isolated macrophages or monocytes, etc. It contains non-infected target cells stained with CFSE mixed with effector cells and stained with 7-AAD to determine spontaneous cell death.

16.3 Run Controls D and E to ensure apoptotic, orange-red fluorescing target cells fall in the proper region. Control D will also determine the basal level of apoptosis that normally occurs within the cell line without the influence of effector cells.

16.4 Run Controls F and G to ensure dead, red fluorescing target cells fall in the proper region. Control F will also determine the level of spontaneous cell death that normally occurs within the cell line without the influence of effector cells.

16.5 Run Control H to determine the background levels of necrosis and apoptosis without the influence of effector cells.

17. Label Samples with 7-AAD

17.1 Add 20 µL 21X 7-AAD to the experimental samples (at 400 µL) and mix or gently vortex.

17.2 Incubate 10 mins on ice protected from light and analyze as soon as possible (Section 14).

18. Flow Cytometry Analysis

18.1 Distinguish green target cells from unstained effector cells using FSC vs. SSC or CFSE (FL-1) vs. SSC

18.2 Create a plot of CFSE (FL-1) vs. 7-AAD (FL-3) and gate on the green CFSE stained target cells (R3).

18.3 From this population of green target cells, prepare a dot plot of SRFLICA(FL-2) vs. 7-AAD (FL-3).

18.4 Calculate total cytotoxicity by adding the percentage of early apoptotic cells in the R5 region, late apoptotic cells in R6, and necrotic cells in R7.

Once the green target cells have been gated (R3), derive a plot of the poly caspase apoptosis reagent (SR-VAD-FMK) in FL-2 vs. live/dead stain (7-AAD) in FL-3. This plot reveals 4 populations of cells: live cells and 3 populations of cells in the death process, including cells in early apoptosis which are not detectable by any other method. All apoptotic events can now be included in the calculation of total cytotoxicity, leading to more accurate results.

Identify green target cells

Run the samples. Derive a FSC vs. SSC plot or CFSE (FL-1) vs. SSC to identify the target cells. Create a plot of CFSE (FL-1) vs. 7-AAD (FL-3) to further distinguish the green target cells from the unstained effector cells. Identify all green target cells and gate on them as R3 (R1+R2=R3). Set acquisition to collect events within R3. Traditional enzyme-release assays are often skewed by the large number of necrotic effector cells.

Quantitate 4 populations

Live Cells: R4 Lower left: Viable live cells are SR-VAD-FMK apoptosis negative and 7-AAD live/dead negative.

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.

Late Apoptosis: R6 Upper right: Late apoptotic cells are SR-VAD-FMK apoptosis positive and 7-AAD live/dead positive.

Necrosis: R7 Upper left: Necrotic cells are SR-FLICA apoptosis negative and 7-AAD live/dead positive.

Calculate total cytotoxicity

Based on the percentage of cells in each quadrant, total cytotoxicity can be calculated.

Total Cytotoxicity = Early apoptosis (R5) + Late apoptosis (R6) + Necrosis (R7)

19. Notes

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