Metabolic Activity/Annexin V/Dead Cell Apoptosis Kit with C_{12} Resazurin, APC annexin V, and SYTOX® Green for Flow Cytometry

Catalog no. V35114

Table 1. Contents and storage information.

Material	Amount	Composition	Storage*	Stability
Allophycocyanin (APC) annexin V (Component A)	250 µL	Solution in 25 mM HEPES, 140 mM NaCl, 1 mM EDTA, pH 7.4, 0.1% bovine serum albumin (BSA)	• 2–6°C • Protect from light • DO NOT FREEZE COMPONENT A	When stored as directed this kit is stable for 6 months.
C_{12}-Resazurin (MW=398, Component B)	40 µg	Dried material		
SYTOX® Green Stain (Component C)	100 µL	10 µM solution in DMSO		
Dimethylsulfoxide (DMSO, Component D)	1.5 mL	High-quality anhydrous DMSO		
5X annexin-binding buffer (Component C)	15 mL	50 mM HEPES, 700 mM NaCl, 12.5 mM $CaCl_2$, pH 7.4		

*For long-term storage, store the vial of C_{12}-Resazurin and SYTOX® Green stain at ≤–20°C. The APC annexin V and SYTOX® Green stain are light sensitive and may be handled in normal room light, but avoid prolonged exposure to light.

Number of assays: Sufficient material is supplied for 50 flow cytometry assays based on a 100 µL assay volume.

Approximate fluorescence excitation/emission maxima: APC annexin V: 650/660 in nm; SYTOX® Green 488**:** 503/524 in nm, bound to DNA; C_{12}-Resazurin: 571/585 in nm.

Introduction

Apoptosis is a carefully regulated process of cell death that occurs as a normal part of development. Inappropriately regulated apoptosis is implicated in disease states, such as Alzheimer's disease and cancer. Apoptosis is distinguished from necrosis by characteristic morphological and biochemical changes, including compaction and fragmentation of the nuclear chromatin, shrinkage of the cytoplasm, and loss of membrane asymmetry.[1-5] In normal viable cells, phosphatidylserine (PS) is located on the cytoplasmic surface of the cell membrane. However, in apoptotic cells, PS is translocated from the inner to the outer leaflet of the plasma membrane, thus exposing PS to the external cellular environment.[6] In leukocyte apoptosis, PS on the outer surface of the cell marks the cell for recognition and phagocytosis by macrophages.[7,8] The human vascular anticoagulant, annexin V, is a 35–36 kilodalton Ca^{2+}-dependent phospholipid-binding protein that has a high affinity for PS.[9] Annexin V labeled with a fluorophore or biotin can identify apoptotic cells by binding to PS exposed on the outer leaflet.[10]

The Metabolic Activity/Annexin V/Dead Cell Apoptosis Kit with C_{12} Resazurin, APC annexin V, and SYTOX® Green for flow cytometry provides a rapid and convenient assay for

apoptosis. The kit contains recombinant annexin V conjugated to allophycocyanin (APC). APC is an extremely fluorescent phycobiliprotein, which can be easily excited with a helium-neon (HeNe) laser at 633 nm. Nonfluorescent C_{12}-resazurin is reduced by viable cells to orange-fluorescent C_{12}-resorufin. Resazurin has been used extensively to detect the metabolic activity of many different cell types, from bacteria to higher eukaryotes.[11-13] The SYTOX® Green dye is impermeant to live and early apoptotic cells, but stains dead cells with intense green fluorescence by binding to cellular nucleic acids. After staining a cell population with APC annexin V, C_{12}-resazurin, and SYTOX® Green stain, apoptotic cells show far-red fluorescence, intermediate orange fluorescence, and no green fluorescence. Dead cells show intense far-red and green fluorescence, and little orange fluorescence. Live cells show little or no green or far-red fluorescence, but show significant fluorescence in the orange channel (Figure 1). These populations can easily be distinguished using a flow cytometer with both the 488 nm line of an argon-ion laser and the 633 nm line of a HeNe laser for excitation.

The assay has been optimized using Jurkat cells, a human T-cell leukemia clone, treated with camptothecin to induce apoptosis. Some modifications may be required for use with other cell types. Since no single parameter defines apoptosis in all systems, it is prudent to use a combination of different measurements for reliable detection of apoptosis. Refer to our website at probes.invitrogen.com for a wide selection of products for apoptosis research.

Before Starting

Materials Required but Not Provided

- Samples (appropriate sample concentrations range from 2×10^5 to 1×10^6 cells/mL)
- Inducing agent
- 2 mM hydrogen peroxide
- Phosphate buffered saline (PBS)
- Deionized water

Caution

No data are available addressing the mutagenicity or toxicity of SYTOX® Green stain. Because this reagent binds to nucleic acids, treat it as a potential mutagen and handle with appropriate care. Handle the DMSO stock solution with particular caution as DMSO is known to facilitate the entry of organic molecules into tissues. Dispose of stains in compliance with all pertaining local regulations.

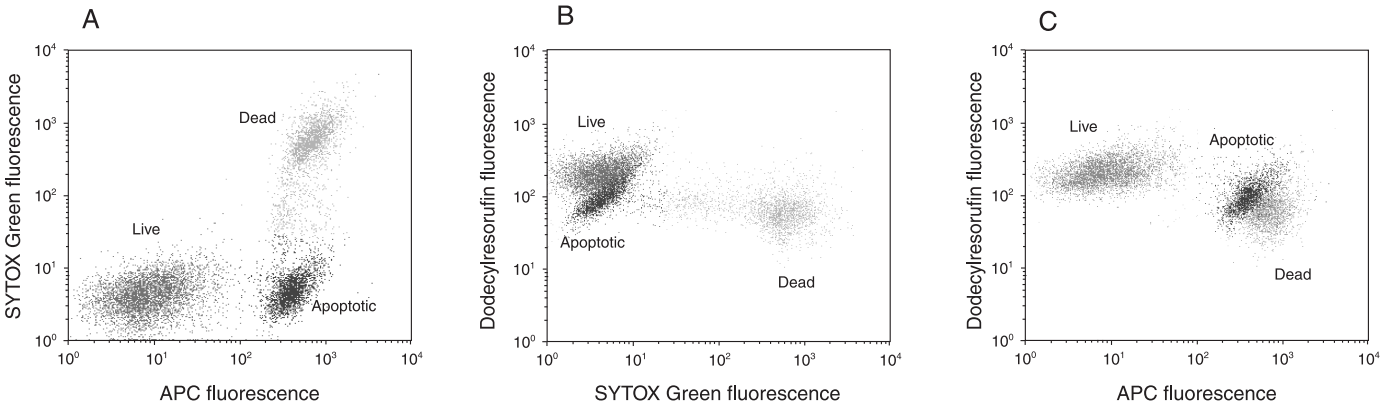

Figure 1. Jurkat cells (T-cell leukemia, human) were treated with either 10 µM camptothecin or 2 mM hydrogen peroxide for 4 hours at 37°C, 5% CO_2, and then mixed together. Cells were incubated with the reagents in the kit, and then analyzed by flow cytometry. The SYTOX® Green fluorescence versus APC (allophycocyanin) fluorescence dot plot (A) shows resolution of live, apoptotic, and dead cell populations. The cell populations can be evaluated for metabolic activity using the resorufin fluorescence versus SYTOX® Green fluorescence and resorufin fluorescence versus APC fluorescence dot plots (B and C).

Experimental Protocol

The assay has been optimized using Jurkat cells treated with camptothecin to induce apoptosis. Some modifications may be required for use with other cell types.

1. Induce apoptosis in cells using the desired method. Prepare a negative control by incubating cells in the absence of inducing agent. Prepare a positive control for necrosis by incubating cells with 2 mM hydrogen peroxide for 4 hours at 37°C.

2. Prepare 1X annexin-binding buffer. For example, for about 10 assays, add 2 mL 5X annexin-binding buffer (Component C) to 8 mL deionized water.

   Note: Prior to opening, warm vials for Components B, C, and D to room temperature. Reseal these vials tightly before returning to storage.

3. Prepare a 1 mM stock solution of C_{12}-resazurin by dissolving the contents of the vial of C_{12}-resazurin (Component B) in 100 µL DMSO (Component D). It may be necessary to agitate the solution in an ultrasonic water bath to fully dissolve the C_{12}-resazurin. The C_{12}-resazurin stock solution is stable for 3 months if stored at ≤–20°C, protected from light.

   Prepare a fresh 50 µM working solution of C_{12}-resazurin by diluting 1 µL of the 1 mM C_{12}-resazurin stock solution in 19 µL DMSO.

4. Prepare a 1 µM working solution of SYTOX® Green stain.

   For example, dilute 5 µL 10 µM SYTOX ® Green stain stock solution (Component C) in 45 µL 1X annexin-binding buffer. The SYTOX ® Green stain concentration may have to be adjusted for individual cell types.

   Store the unused portion of this working solution at ≤–20°C for up to 1 month.

5. Harvest the cells following apoptosis induction and wash in 1X annexin-binding buffer.

6. Pellet the washed cells (from step 5) by centrifugation, discard the supernatant, and resuspend the cells at a concentration of ~1×10^6 cells/mL in 1X annexin-binding buffer.

7. Add 5 µL of APC annexin V (Component A), 1 µL of the 50 µM C_{12}-resazurin working solution (prepared in step 3), and 1 µL of the 1 µM SYTOX® Green stain working solution (prepared in step 4) to each 100 µL of cell suspension.

8. Incubate the cells at 37°C in an atmosphere of 5% CO_2 for 15 minutes.

9. After the incubation period, add 400 µL 1X annexin-binding buffer, mix gently, and keep the samples on ice.

10. As soon as possible, analyze the stained cells by flow cytometry, measuring the fluorescence emission at 530 nm and 575 nm using 488 nm excitation, and at 660 nm using 633 nm excitation. The population should separate into three groups: live cells with only a low level of green and far-red fluorescence and a high level of orange fluorescence; apoptotic cells with a high level of far-red fluorescence, intermediate orange fluorescence, and no green fluorescence; and dead cells with a high level of green and far-red fluorescence and a low level of orange fluorescence. Confirm the flow cytometry results by viewing the cells with a fluorescence microscope, using filters appropriate for fluorescein (FITC), tetramethylrhodamine (TRITC), and allophycocyanin (APC).

References

1. Immunol Cell Biol 76, 1 (1998); **2.** Cytometry 27, 1 (1997); **3.** J Pharmacol Toxicol Methods 37, 215 (1997); **4.** FASEB J 9, 1277 (1995); **5.** Am J Pathol 146, 3 (1995); **6.** Cytometry 31, 1 (1998); **7.** J Immunol 148, 2207 (1992); **8.** J Immunol 151, 4274 (1993); **9.** J Biol Chem 265, 4923 (1990); **10.** Blood 84, 1415 (1994); **11.** Appl Environ Microbiol 56, 3785 (1990); **12.** J Dairy Res 57, 239 (1990); **13.** J Neurosci Methods 70, 195 (1996).

Product List **Current prices may be obtained from our website or from our Customer Service Department.**

Cat. no.	Product Name	Unit Size
V35114	Metabolic Activity/Annexin V/Dead Cell Apoptosis Kit with C_{12} Resazurin, APC annexin V, and SYTOX® Green *flow cytometry* *50 assays*	1 kit

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