Multi-Parameter Apoptosis Assay Kit

Item No. 600330

Customer Service 800.364.9897 * **Technical Support** 888.526.5351
www.caymanchem.com

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -20°C kit. For best results, remove components and store as stated below.

Item Number	Item	Quantity/Size	Storage
600301	Cell-Based Annexin V FITC	1 vial/50 µl	4°C
600302	Cell-Based Assay Annexin V Binding Buffer (10X)	1 vial/50 ml	RT
400201	Cell-Based Assay 7-AAD Staining Stock Solution (1,000X)	1 vial/50 µl	4°C
600331	Cell-Based Assay TMRE	1 vial/100 µl	-20°C
600332	Cell-Based Assay Hoechst Dye	1 vial/50 µl	4°C

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3641

Email: techserv@caymanchem.com

Hours: M-F 8:00 AM to 5:30 PM EST

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section, on page 3, and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. Adjustable pipettes and a repeating pipettor
2. 6-, 12-, 24-, or 96-well plates for culturing cells
3. Flow cytometer, fluorescence microscope, or plate reader equipped with a laser or filters capable of detecting TMRE at excitation wavelengths between 520-570 nm and emission wavelengths between 570-610 nm, FITC at excitation/emission = 485/535 nm, 7-AAD at excitation/emission = 555/655 nm, and Hoechst Dye at excitation/emission = 355/465 nm
4. A plate centrifuge

INTRODUCTION

Background

Apoptosis is a programmed, well controlled process of cell death in which a sequence of biochemical events leads to characteristic changes in cell morphology and eventually cell death. These changes include loss of cell membrane asymmetry, cell shrinkage, membrane blebbing, nuclear fragmentation, and chromatin condensation. During an organism's life cycle, apoptosis plays a vital role in normal development and maintenance of tissue homeostasis by eliminating old, unnecessary, and unhealthy cells. Dysregulation of apoptosis results in pathological conditions including neurodegenerative diseases, ischemic damage, autoimmune disorders, and cancer.[1] The ability to modulate cell fate (life or death) is thus recognized as an immense therapeutic potential in drug discovery. Research continues to focus on the elucidation and analysis of signaling pathways that control apoptosis.

One of the hallmarks of the early stages of apoptosis is that membrane phospholipids such as phosphatidylserine and phosphatidylethanolamine redistribute from the inner to outer leaflet of the membrane bilayer where they are exposed on the cell surface.[2,3] Externalization of phosphatidylserine residues to the outer plasma membrane leaflet allows its detection *via* its high-affinity for annexin V, a phospholipid binding protein. Apoptotic cells bound with fluorochrome-labeled Annexin V can be visualized using fluorescence microscopy, flow cytometry or a plate reader capable of fluorescence measurements. Compared to other apoptosis detection methods, such as TUNEL (TdT-mediated dUTP Nick-End Labeling) analysis, ISEL (*in situ* end labeling), and DNA laddering analysis for detection of fragmentation of DNA, Annexin-V analysis has advantages of being more sensitive and less time consuming.

Another key event that occurs during apoptosis is the loss of mitochondrial transmembrane potential. For this reason, mitochondrial transmembrane potential has been used as an indicator of cell viability. Tetramethylrhodamine ethyl ester (TMRE) is a lipophilic rhodamine probe that has been widely used to measure mitochondrial potential in live cells.

About This Assay

Cayman's Multi-Parameter Apoptosis Assay Kit employs FITC-conjugated Annexin V as a probe for phosphatidylserine on the outer membrane of apoptotic cells, TMRE as a probe for mitochondrial membrane potential, 7-AAD as an indicator of membrane permeability/cell viability, and Hoechst Dye to demonstrate nuclear morphology. The kit allows phenotypic characterization of different cell death parameters at a single-cell level. The assay can be adapted to high content screening with appropriate equipment. The reagents provided in the kit are sufficient to run 100 samples when using flow cytometry, or 500 samples when using a 96-well plate format.

PRE-ASSAY PREPARATION

NOTE: All the fluorescence reagents are light sensitive. Do not expose to direct intense light. You may not need the 7-AAD Stock Solution if you are using a microscope that does not have a filter set to detect an excitation/emission = 555 nm/655 nm. You may not need the Hoechst Dye if you are using a flow cytometer that is not equipped with a UV laser.

Reagent Preparation

Binding Buffer (1X)

Prepare a working binding buffer by diluting the Cell-Based Assay Annexin V Binding Buffer (10X) (Item No. 600302) 1:10 in distilled water (for example, add 10 ml of the Cell-Based Assay Annexin V Binding Buffer (10X) to 90 ml of distilled water). Mix well and keep at room temperature. The diluted Binding Buffer will be stable for one year at room temperature.

Annexin V FITC/7-AAD Staining Solution

Prepare an Annexin V FITC/7-AAD Staining Solution by adding 10 µl of Cell-Based Annexin V FITC (Item No. 600301) and 5 µl of Cell-Based Assay 7-AAD Staining Stock Solution (1,000X) (Item No. 400201) to 5 ml of Binding Buffer. Mix well. Prepare this staining solution immediately before adding to the samples. Protect from light. Omit adding the Cell-Based Assay 7-AAD Staining Stock Solution (1,000X) if you are using a microscope that is not equipped with a filter for detecting excitation/emission = 555 nm/655 nm. The Annexin V FITC/7-AAD Staining Solution will be stable for one hour at 4°C. *NOTE: Protect from light.*

TMRE/Hoechst Dye Staining Solution

Prepare a TMRE/Hoechst Dye Staining Solution by adding 10 µl of Cell-Based Assay TMRE (Item No. 600331) and 5 µl of Cell-Based Assay Hoechst Dye (Item No. 600332) to 1 ml of the culture medium used in your experiment. Mix well and protect from light. Omit adding the Cell-Based Assay Hoechst Dye if you are using a flow cytometer that does not have a UV laser. The TMRE/Hoechst Dye Staining Solution will be stable for one hour at 4°C. *NOTE: Protect from light.*

ASSAY PROTOCOL

NOTES

- All the fluorescence reagents are light sensitive. All staining procedures must be performed without direct exposure to intense light. Incubations should be done in the dark.
- For all assay protocols described below, it is imperative that samples be analyzed immediately following completion of the staining.

Flow Cytometry

1. Culture cells in 6-, 12-, or 24-well plates at a density of 5 x 10^5 cells/ml in a CO_2 incubator overnight at 37°C. Treat the cells with experimental compounds or vehicle (each sample should be run in duplicate or triplicate). Incubate the cells according to your normal protocol.
2. Add 100 µl of the TMRE/Hoechst Dye Staining Solution per ml of culture medium to each well of the plate. For example, if you culture cells in 2 ml of culture medium in a 6-well plate, add 200 µl of the Hoechst Dye Staining Solution into each well. Mix gently.
3. Incubate samples in a CO_2 incubator at 37°C for 10-30 minutes. Sufficient staining is usually obtained after 10 minutes of incubation.

   NOTE: You may take a subset of sample at this point to measure the flourescence of TMRE staining (excitation/emission = 560 nm/595 nm, usually the FL2 channel of a flow cytometer). The TMRE staining intensity may decrease or diminish in the subsequent steps.
4. Collect the cells in a test tube and centrifuge at 400 x g for five minutes. Aspirate and discard the supernatant.
5. Resuspend the cells with 2 ml of diluted Binding Buffer. Mix well to ensure separation of individual cells.
6. Centrifuge the cells at 400 x g for five minutes. Aspirate and discard the supernatant.

7. Resuspend the cells in 250 µl of Annexin V FITC/7-AAD Staining Solution. Mix well to ensure separation of individual cells. Incubate the cells in the dark at room temperature for 10 minutes.
8. Centrifuge the cells at 400 x g for five minutes. Aspirate and discard the supernatant.
9. Resuspend the cells in 0.25-0.5 ml of the Binding Buffer. Mix well to ensure separation of individual cells.
10. Analyze the cells with a flow cytometer. The cells must be analyzed immediately. Early stage apoptotic cells recognized by Annexin V FITC are detectable in the FL1 channel. Cells with healthy mitochondria will be stained by TMRE and will be detectable in the FL2 channel. Dead cells are stained by 7-AAD and are detectable in the FL3 channel of a flow cytometer.

Fluorescence Microscopy

A 6-, 12-, 24-, or 96-well culture plate can be used for this method. We recommend that the cell density be ≤1 x 10^6 cells/ml. Optimal conditions will depend on the cell type.

1. Culture cells in 6-, 12-, 24-, or 96-well plates at a density of 5 x 10^5 cells/ml in a CO_2 incubator overnight at 37°C. Treat the cells with experimental compounds or vehicle (each sample should be run in duplicate or triplicate). Incubate the cells according to your normal protocol.
2. Add 100 µl of the TMRE/Hoechst Dye Staining Solution per ml of culture medium to each well of the plate. For example, if you culture cells in 2 ml of culture medium in a 6-well plate, add 200 µl of the Hoechst Dye Staining Solution into each well. Mix gently.
3. Incubate samples in a CO_2 incubator at 37°C for 10-30 minutes. Sufficient staining is usually obtained after 10 minutes of incubation.

   NOTE: You may observe the flourescence of TMRE staining (excitation/emission = 560 nm/595 nm) at this point, as the staining intensity may decrease or diminish in the subsequent steps.
4. Centrifuge the cells at 400 x g for five minutes. Carefully aspirate and discard the supernatant.
5. Add 2 ml, 1 ml, 500 µl, or 100 µl of Binding Buffer to each well of a 6-, 12-, 24-, or 96-well plate, respectively.
6. Centrifuge the plate for five minutes at 400 x g at room temperature. Carefully aspirate and discard the supernatant.
7. Add 800 µl, 400 µl, 200 µl, or 50 µl of Annexin V FITC/7-AAD Staining Solution to each well of the 6-, 12-, 24-, or 96-well plate, respectively. Incubate for 10 minutes at room temperature in the dark.
8. Centrifuge the plate for five minutes at 400 x g at room temperature. Carefully aspirate and discard the supernatant.
9. Add 2 ml, 1ml, 500 µl, or 100 µl of Binding Buffer prepared above to each well of a 6-, 12-, 24-, or 96-well plate, respectively.
10. Examine the cells by fluorescence microscopy. Cells must be analyzed immediately. Cells with healthy mitochondria are stained by TMRE and can be detected using a filter designed to detect rhodamine (excitation/emission = 540/570 nm) or Texas Red (excitation/emission = 590/610 nm). Early stage apoptotic cells stained by Annexin V FITC can be detected using a filter designed to detect fluorescein (excitation/emission = 485/535). Hoechst Dye staining of cell nuclei can be visualized with a UV filter.

Plate Reader Fluorescence Detection

A 96-well **black** culture plate should be used for this method. We recommend that the cell density be 1 x 10^5-1 x 10^6 cells/ml. Optimal conditions will depend on the cell type.

1. Culture cells in a 96-well black plate at a density of 1 x 10^5-5 x 10^5 cells/well in a CO_2 incubator overnight at 37°C. Treat the cells with experimental compounds or vehicle (each sample should be run in duplicate or triplicate). Incubate the cells according to your normal protocol.
2. Add 100 µl of the TMRE/Hoechst Dye Staining Solution per ml of culture medium to each well of the plate. For example, if you culture cells in 100 µl of culture medium in the 96-well black plate, add 10 µl of the TMRE/Hoechst Dye Staining Solution into each well. Mix gently.

3. Incubate samples in a CO_2 incubator at 37°C for 10-30 minutes. Sufficient staining is usually obtained after 10 minutes of incubation.

   NOTE: You may read the plate at this point to measure the fluorescence of TMRE staining (excitation/emission = 560 nm/595 nm), as the staining intensity may decrease or diminish in the subsequent steps.

4. Centrifuge the cells at 400 x g for five minutes. Carefully aspirate and discard the supernatant.
5. Add 200 µl of Binding Buffer to each well and centrifuge the plate for five minutes at 400 x g at room temperature. Carefully aspirate and discard the supernatant.
6. Incubate the cells in 50 µl of the Annexin V FITC/7-AAD Staining Solution for 10 minutes at room temperature.
7. Centrifuge the plate for five minutes at 400 x g at room temperature. Carefully aspirate and discard the supernatant.
8. Add 100 µl of Binding Buffer to each well. The cells are now ready for analysis with a plate reader equipped with appropriate fluorescence capabilities. Cells with healthy mitochondria display strong fluorescence intensity with excitation and emission at 560 nm and 595 nm, respectively. Early stage apoptotic cells stained by Annexin V FITC can be detected with excitation and emission at 485 nm and 535 nm, respectively. Cell number/density will be reflected by the fluorescence intensity with Hoechst Dye staining detectable with excitation and emission at 355 nm and 465 nm, respectively.

ANALYSIS

Performance Characteristics

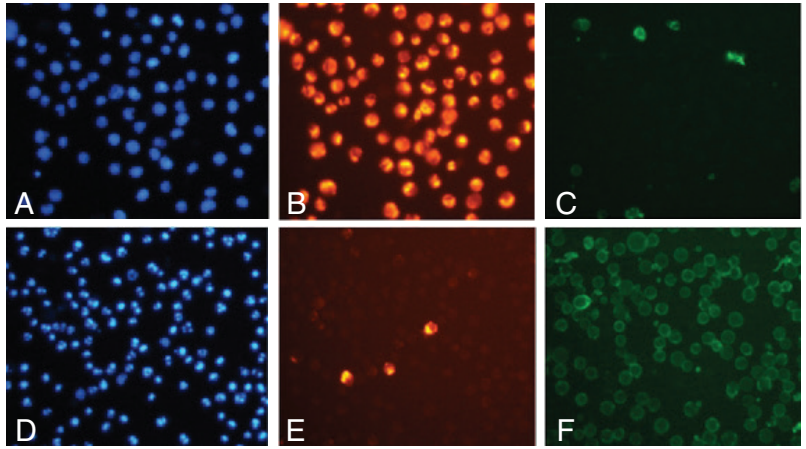

Figure 1. Staurosporine induces apoptosis in Jurkat cells, as measured by nuclear morphology, a decrease in mitochondrial membrane potential, and an increase of Annexin V FITC positive cells. Jurkat cells were plated at a density of 5 x 10^4 cells/well in a 6-well plate. The next day, cells were treated with vehicle (control) or 2.5 µg/ml staurosporine (treatment) for five hours in a CO_2 incubator at 37°C. Cells were then processed for staining of nuclei with Hoechst Dye, mitochondrial membrane potential with TMRE, and Annexin V FITC according to the protocol above. Panels A-C show same field of cells from the control group whereas panels D-F show the same field of cells from the staurosporine-treated group. Hoechst staining shows that most control cells have round and intact nuclei *(Panel A)* whereas staurosporine-treated cells mostly have condensed and fragmented nuclei *(Panel D)*. TMRE staining reveals that most control cells have undisrupted mitochondrial membrane potential *(Panel B)* whereas staurosporine-treated cells mostly have diminished membrane potential and were not stained *(Panel E)*. Most control cells are Annexin V negative *(Panel C)* whereas cells treated with staurosporine are mostly Annexin V positive *(Panel F)*, indicating cells are undergoing apoptosis.

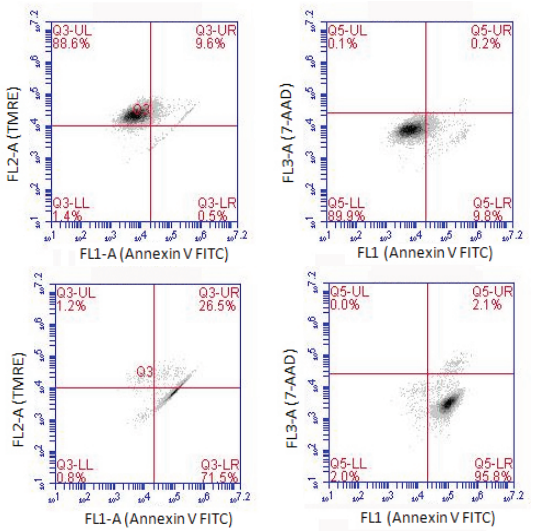

Figure 2. Staurosporine induces apoptosis in Jurkat cells as measured by flow cytometry. Jurkat cells were plated at a density of 1 x 10^6 cells/ml in a 6-well plate. The next day, cells were treated with vehicle (top two panels), or 2.5 µg/ml staurosporine for five hours in a CO_2 incubator at 37°C (bottom two panels). Cells were then processed for staining according to the protocol above. A majority of control cells were strongly stained by TMRE (top left panel, top left quadrant), but not stained with either Annexin V FITC or 7-AAD (top right panel, top right, and bottom right quadrants). When cells were treated with 2.5 µg/ml staurosporine, most of cells lost mitochondrial membrane potential, evident by significant decrease in TMRE staining intensity (bottom left panel, top left quadrant). Instead, most of cells were Annexin V FITC positive (bottom right panel, lower right quadrant) and some of these cells were dead cells (bottom right panel, top right quadrant).

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Strong staining for both Annexin V FITC and 7-AAD in all samples, including controls	Cells are not healthy	Use only healthy cells
High level of Annexin V FITC staining in all samples, including controls	Cells are damaged during harvesting or processing for staining	Process the sample gently; for example, disperse cells gently by pipetting cells up and down; Do not vortex the cells; Do not use scraping for adherent cells
No signal for Annexin V FITC	A. Annexin V FITC/7-AAD Staining Solution not prepared properly B. Cells lost during processing	A. Use right amount of Annexin V FITC to prepare Annexin V FITC/7-AAD Staining Solution B. Decrease treatment time or compound dosage
Very strong TMRE staining	TMRE concentration too high for your cells	Further dilute TMRE solution

References

1. Schiller, M., Bekerdjian-Ding, I., Heyder, P., *et al.* Autoantigens are translocated into small apoptotic bodies during early stages of apoptosis. *Cell Death and Differentiation* **15**, 183-191 (2008).
2. van Engeland, M., Nieland, L.J.W., Ramaekers, F.C.S., *et al.* Annexin V-affinity assay: A review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry* **31**, 1-9 (1998).
3. Elmore, S. Apoptosis: A review of programmed cell death. *Toxicol. Pathol.* **35(4)**, 495-516 (2007).

NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.