Early Apoptosis Detection Assay Kit

Item No. 601360

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a 4°C kit. After opening kit, store individual components as stated below.

Item Number	Item	Quantity/Size	Storage
601281	Annexin V FITC Reagent	1 vial/100 tests	4°C
600302	Cell-Based Assay Annexin V Binding Buffer (10X)	1 vial/50 ml	4°C
601361	DAPI Viability Dye	1 vial/100 µl	4°C
601283	TMRE Dye	1 vial/50 nmol	4°C
601362	TO-PRO®-3 Dye	1 vial/25 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. DMSO
2. PBS, pH 7.4
3. Flow cytometer equipped with lasers and filters capable of exciting and detecting the wavelengths shown in Table 1.
4. A plate centrifuge

INTRODUCTION

Background

Apoptosis is a programmed, non-inflammatory 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 and integrity, 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.

In order for apoptotic cell death to be a non-inflammatory process, cell debris must be removed without triggering an immune response. Thus, cells undergoing apoptosis release "find me" and "eat me" signals to induce their own phagocytosis. These signals include ATP and UTP, which are released through the selective channel pannexin 1 when it is activated.[2] This caspase-dependent event occurs early in apoptosis, and can be detected by staining with monomeric cyanine dyes such as TO-PRO®-3.[3] Using this dye in combination with the traditional annexin V membrane lipid asymmetry probe, researchers can classify cells as early or late apoptotic and also distinguish apoptotic bodies from debris.

A critical component of the apoptotic cascade is the loss of mitochondrial membrane potential ($\Delta_{\psi m}$). Tetramethylrhodamine ethyl ester (TMRE) is a lipophilic rhodamine probe that is sequestered in intact mitochondria, and dissipated when $\Delta_{\psi m}$ is lost during apoptosis.[4] This property makes TMRE useful for the flow cytometric detection of perturbations in $\Delta_{\psi m}$.

About This Assay

Cayman's Early Apoptosis Detection Assay Kit employs FITC-conjugated annexin V as a probe for phosphatidylserine (PS) on the outer membrane of apoptotic cells, TMRE as a probe for $\Delta\psi_m$, DAPI as an indicator of membrane permeability/cell viability, and TO-PRO®-3 to indicate the selective permeability of pannexin channels. The Early Apoptosis Detection Assay Kit allows phenotypic characterization of multiple different cell death parameters at the single-cell level. The reagents provided in the kit are sufficient to run 100 samples.

Reagent	Flow cytometric laser/filter sets (nm)	What it stains
DAPI	350 (or 405)/450	Nuclei of dead cells only (plasma membrane permeability)
Annexin V FITC	488/525	PS flipped to outside of plasma membrane
TMRE	488/585	Mitochondria with high membrane potential
TO-PRO®-3	633/700	Nuclei of dead cells and cells with active pannexin channels

Table 1. Dye combination included in this kit and flow cytometer requirements

PRE-ASSAY PREPARATION

NOTE: This protocol is designed to be modular, as any of the fluorophores can be excluded if your experimental setup does not require them or cannot detect them.

Reagent Preparation

TMRE Solution

Reconstitute TMRE Dye (Item No. 601283) with 100 µl of DMSO. Mix well. After use, this TMRE Solution can be stored at -20°C and will be stable for up to six months.

1X Binding Buffer

Dilute Cell-Based Assay Annexin V Binding Buffer (10X) (Item No. 600302) 1:10 in distilled water. This solution will be stable for one year at 4°C.

Staining Solution

Prepare a Staining Solution by adding 2.5 µl of TMRE Solution, 2 µl of TO-PRO®-3 Dye (Item No. 601362), and 25 µl Annexin V FITC Reagent (Item No. 601281) to 5 ml of 1X Binding Buffer prepared above. Mix well, protect from light, and use within one hour.

DAPI Staining Solution

Dilute DAPI Viability Dye (Item No. 601361) 1:100-1:200 in PBS, pH 7.4. This solution will be stable for one hour at room temperature, *protected from light*.

ASSAY PROTOCOL

NOTE: It is recommended for flow cytometric applications to prepare single-stained control samples for the purposes of fluorescence compensation.

Suspension Cells

1. Culture cells and treat as required by your experimental design in a CO_2 incubator at 37°C, testing each sample in duplicate or triplicate. For shorter duration experiments, the entire protocol can be modified to be performed in v-bottom polypropylene staining plates.
2. Collect 1-5 x 10^5 cells by centrifugation at 400 x g for five minutes and discard supernatant.
3. Resuspend the cells in 100 µl of Staining Solution prepared on page 8. Mix well to ensure separation of individual cells.
4. Incubate samples at room temperature in the dark for 15 minutes.
5. Centrifuge at 400 x g for five minutes. Carefully remove the supernatant.
6. Resuspend the cells in 100-200 µl of DAPI Staining Solution. Mix well to ensure separation of individual cells.
7. Analyze the cells with a flow cytometer using the channels shown in Table 1 on page 7. The cells must be analyzed immediately.

Performance Characteristics

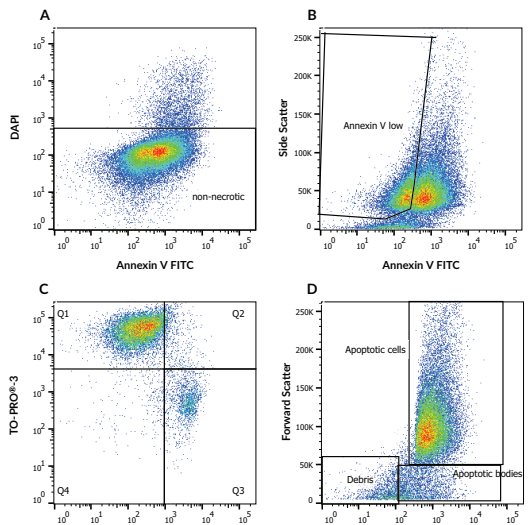

Figure 1. Apoptosis induced by ultraviolet light in Jurkat cells, analyzed by flow cytometry. Jurkat cells were treated with 200 mJ/cm^2 ultraviolet light (UV), then incubated for four hours at 37°C. Cells were then processed according to the suspension cell protocol in this kit booklet. Data were collected by a MACSQuant® flow cytometer from Miltenyi and analyzed using FlowJo® software. For analysis, live cells were gated as DAPI-negative (*Panel A*). The annexin V low cells (*Panel B*) were visualized within a TO-PRO®-3 *versus* TMRE dot plot (*Panel C*), in which TMRE low, TO-PRO®-3 high cells (Q1) are early apoptotic while viable cells are TMRE high, TO-PRO®-3 low (Q3). The remaining events from *Panel B* can be separated into late apoptotic cells (annexin V positive, FSC high), apoptotic bodies (annexin V positive, FSC low) and debris (annexin V negative) (*Panel D*).

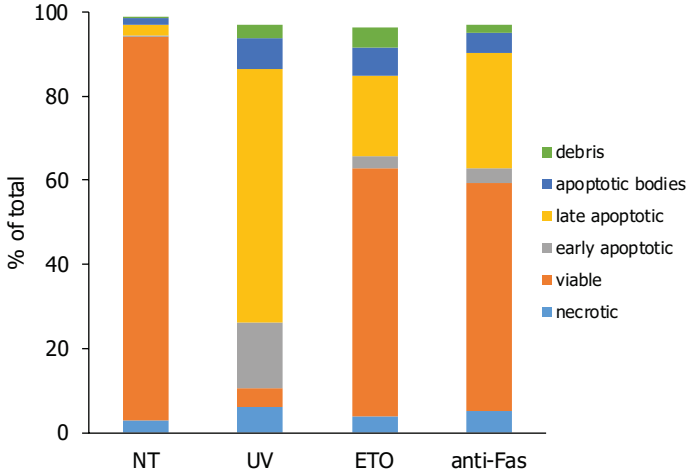

Figure 2. Segmentation of cell populations induced to undergo apoptosis by various stimuli. Jurkat cells were left untreated (NT) or stimulated with 200 mJ/cm^2 ultraviolet light (UV), 50 µM etoposide (ETO), or 50 ng/ml anti-Fas CH-11 for four hours prior to processing for flow cytometry as described in this kit booklet. The percentage of the total population in each segment was quantified as described in Figure 1.

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Strong staining for both annexin V FITC and DAPI in all samples, including controls	A. Cells are damaged during harvesting or processing for staining B. Cells are not healthy	A. Process the sample gently B. Use only healthy cells
No cells seen	Cells lost during processing	Decrease treatment time or compound dosage
No signal for annexin V FITC	A. Not enough annexin V FITC in staining solution B. Cell death stimulus not potent enough	A. Titrate annexin V FITC for each cell type used B. Use stronger/positive control death stimulus
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 Differ.* **15**, 183-191 (2008).
2. F. B. Chekeni, M. R. Elliott, J. K. Sandilos, *et al.* Pannexin 1 channels mediate 'find-me' signal release and membrane permeability during apoptosis. *Nature* **467(7317)**, 863-867 (2010).
3. L. Jiang, R. Tixeira, S. Caruso, *et al.* Monitoring the progression of cell death and the disassembly of dying cells by flow cytometry. *Nat. Protoc.* **11(4)**, 655-663 (2016).
4. T. Jurikova, O. Rop, J. Mlcek, *et al.* Phenolic profile of edible honeysuckle berries (genus *Lonicera*) and their biological effects. *Molecules* **17(1)**, 61-79 (2011).

NOTES

Warranty and Limitation of Remedy

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