Multi-Parameter Apoptosis Assay Kit

Item No. 601280

www.caymanchem.com
Customer Service 800.364.9897
Technical Support 888.526.5351
1180 E. Ellsworth Rd · Ann Arbor, MI · USA

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a 4°C kit. Once opened, please remove components and store as stated below.

Item Number	Item	Quantity/Size	Storage
601281	Annexin V FITC Reagent	1 vial	4°C
600302	Cell-Based Assay Annexin V Binding Buffer (10X)	1 vial/50 ml	RT
601282	RedDot™2 Viability Dye	1 vial/50 µl	4°C
601283	TMRE Dye	1 vial	4°C
600332	Cell-Based Assay Hoechst Dye	1 vial/50 µl	4°C

NOTE: RedDot™2 is a product of Biotium, Inc.

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 or fluorescence microscope equipped with lasers and filters capable of exciting and detecting the wavelengths shown in Table 1 on page 6
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.

One of the hallmarks of apoptosis is the redistribution of membrane phospholipids such as phosphatidylserine (PS) and phosphatidylethanolamine from the inner to the outer leaflet of the membrane bilayer where they are exposed on the cell surface.[2,3] Externalization of PS residues to the outer plasma membrane leaflet allows their detection *via* high-affinity for annexin V, a phospholipid binding protein. Apoptotic cells bound by fluorochrome-labeled annexin V can be visualized using fluorescence microscopy or flow cytometry.

Another 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 or microscopic detection of perturbations in $\Delta_{\psi m}$.

At later stages of apoptosis, the plasma membrane starts to break down and membrane-impermeable dyes like RedDot™2 gain access to the cytosol and nucleus, where they fluoresce upon binding to DNA.[5] In addition, the nuclear DNA condenses and then finally fragments, which can be morphologically detected using sensitive DNA dyes like Hoechst 33342.[6]

About This Assay

Cayman's Multi-Parameter Apoptosis Assay Kit employs FITC-conjugated Annexin V as a probe for PS on the outer membrane of apoptotic cells, TMRE as a probe for $\Delta_{\psi m}$, RedDot™2 as an indicator of membrane permeability/cell viability, and Hoechst Dye to demonstrate nuclear morphology. The 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	Ex/Em Filters	Flow cytometric laser/filter sets	What it stains
Hoechst 33342	350/461	350 (or 405)/450	All nuclei (condensed chromatin)
Annexin V FITC	490/517	488/525	PS redistributed to external plasma membrane by apoptotic cells
TMRE	540/595	488/585	Mitochondria with intact membrane potential
RedDot™2	646/697	633/700	Nuclei of dead cells only (plasma membrane permeability)

Table 1. Dye combination included in this kit and optimal detection methods for fluorescence microscopy and flow cytometry

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 should 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 should be stable for three months at 4°C.

Staining Solution

Prepare a Staining Solution by adding 2.5 µl of TMRE Solution, 2 µl of Cell-Based Assay Hoechst Dye (Item No. 600332), 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.

RedDot™2 Staining Solution

Dilute RedDot™2 Viability Dye (Item No. 601282) 1:200 in PBS, pH 7.4. This solution should be stable for one hour at room temperature.

Alternative Staining Solution

If performing the alternative staining protocol for fluorescence microscopy on page 10, prepare the Alternative Staining Solution by adding 2.5 µl of TMRE Solution, 2 µl of Cell-Based Assay Hoechst Dye (Item No. 600332), and 25 µl RedDot™2 Viability Dye (Item No. 601282) to 5 ml PBS, pH 7.4.

ASSAY PROTOCOL

NOTE: We recommend analyzing suspension cells by flow cytometry and adherent cells by fluorescence microscopy. Removing most adherent cell lines from the culture dish by any method is incompatible with this staining protocol.

Suspension Cells

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

1. Culture cells and treat as required by your experimental design in a CO_2 incubator at 37°C, running 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 x 10^5 to 1 x 10^6 cells 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 7. 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 RedDot™2 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 6. The cells must be analyzed immediately.

Adherent Cells (Fluorescence Microscopy)

NOTES:

A. This protocol is designed for use in a 24-well plate or 4-well chamber slide, with 500 µl per well. For different vessel sizes, adjust volumes accordingly.

B. Many adherent cells present a challenge in this assay, as staining for Annexin V can result in morphological changes in the cells or even release from the culture surface. In this case, a 2-step staining procedure can be undertaken, separating TMRE, RedDot™2, and Hoechst staining from Annexin V staining. This alternative protocol is presented below.

1. Culture cells and treat as required by your experimental design in a CO_2 incubator at 37°C, running each sample in duplicate or triplicate. Cells should be <80% confluent at the time of staining.
2. Remove culture medium from each well, being careful not to disturb cell layer.
3. Add 250 µl of the Staining Solution prepared on page 7 to each well of the plate.
4. Incubate samples at room temperature in the dark for 15 minutes.
5. Carefully remove the Staining Solution and add 500 µl PBS, pH 7.4.
6. Remove PBS and add 250 µl of RedDot™2 Staining Solution per well.
7. Visualize cells on your fluorescence microscope using the filter sets shown in Table 1 on page 6.

Alternative protocol:

1. Culture cells and treat as required by your experimental design in a CO_2 incubator at 37°C, running each sample in duplicate or triplicate. Cells should be <80% confluent at the time of staining.
2. Remove culture medium from each well, being careful not to disturb cell layer.
3. Add 250 µl of the Alternative Staining Solution prepared on page 7 to each well of the plate.
4. Incubate samples in a CO_2 incubator at 37°C for 15 minutes.
5. Carefully remove the Alternative Staining Solution and add 500 µl PBS, pH 7.4.
6. Visualize cells on your fluorescence microscope using the filter sets shown in Table 1 on page 6.
7. To test Annexin V FITC staining, dilute Annexin V FITC reagent 1:100-1:400 in 1X Binding Buffer. Aspirate the PBS from your cells and add 250 µl of the diluted Annexin V FITC.
8. Incubate samples at room temperature in the dark for 15 minutes.
9. Carefully remove the Annexin V staining solution and add 500 µl PBS, pH 7.4.
10. Visualize Annexin V FITC staining on your fluorescence microscope using the filter sets shown in Table 1 on page 6.

ANALYSIS

Performance Characteristics

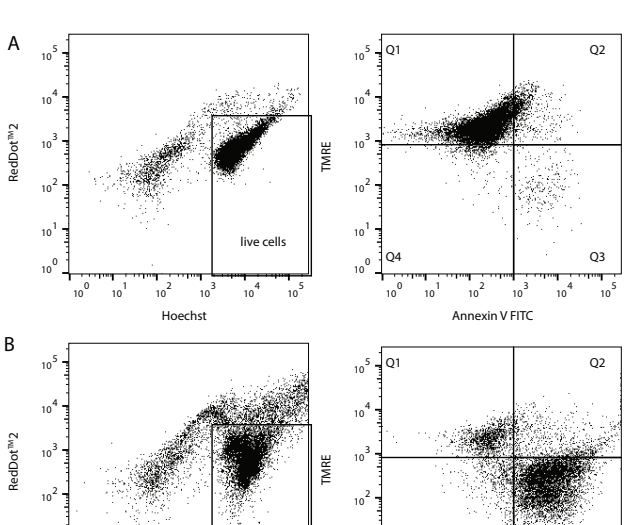

Figure 1. Apoptosis induced by ultraviolet light in Jurkat cells, analyzed by flow cytometry. Jurkat cells were left untreated *(Panel A)*, or treated with 200 mJ/cm^2 ultraviolet light *(Panel B)*, then incubated for four hours at 37°C. Cells were then processed according to the suspension cell protocol in this kit booklet. Data was collected by a MACSQuant® cytometer from Miltenyi and analyzed in FlowJo® software. For analysis, live cells were gated as Hoechst positive, RedDot™2 negative (left dot plots) and visualized for Annexin V FITC and TMRE (right dot plots). As cells undergo apoptosis, they increase in Annexin V FITC staining and decrease in TMRE staining, moving from Q1 to Q3.

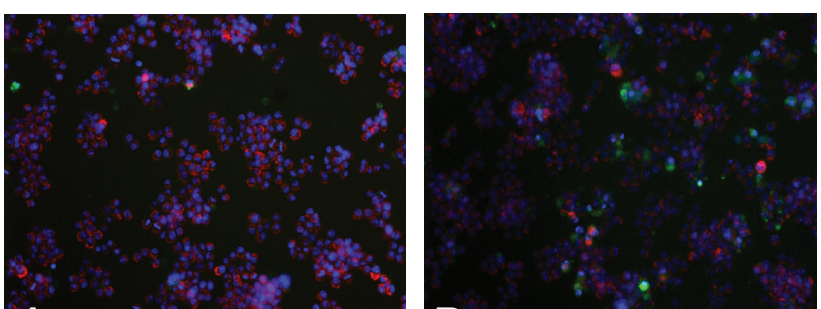

Figure 2. RAW 264.7 cells stimulated to undergo apoptosis by UV concurrently decrease TMRE staining and increase Annexin V FITC staining. RAW 264.7 cells were left untreated *(Panel A)* or stimulated with 200 mJ/cm^2 UV light *(Panel B)*. After a four hour incubation at 37°C, cells were stained according to the adherent cell protocol in this kit booklet. Images were collected on a fluorescence microscope at 200X magnification. Hoechst stain is colored blue, Annexin V FITC is green, and TMRE is red.

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Strong staining for both Annexin V FITC and RedDot™2 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	Titrate 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 Differ.* **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).
4. Perry, S.W., Norman, J.P., Barbieri, J., *et al.* Mitochondrial membrane potential probes and the proton gradient: A practical usage guide. *Biotechniques* **50(2)**, 98-115 (2011).
5. Skindersoe, M.E., and Kjaerulff, S. Comparison of three thiol probes for determination of apoptosis-related changes in cellular redox status. *Cytometry A.* **85(2)**, 179-187 (2014).
6. Telford, W.G., King, L.E., and Fraker, P.J. Comparative evaluation of several DNA binding dyes in the detection of apoptosis-associated chromatin degradation by flow cytometry. *Cytometry* **13(2)**, 137-143 (1992).

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.