General Annexin V Staining Procedure

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Changes from last version			
Date of version	***Paragraphs***	***Date of version***	***Paragraphs***

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Content

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1. Subject

This Standard Operation Procedure (SOP) describes a method to stain mononuclear cells to identify apoptosis at an early stage

2. Application

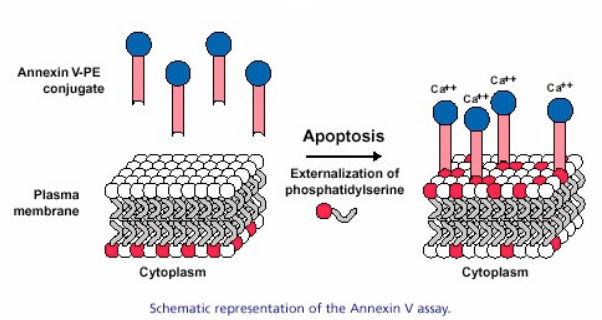
Flow cytometric analysis

3. Definitions and Abbreviations

FBS	= foetal bovine serum (≈ foetal calf serum)
RT	= room temperature
P/S	= Penicillin-Streptomycin
Glu	= L-glutamine
v/v	= volume/volume
rpm	= rotations per minute
ml	= milliliter

4. Principle

PE Annexin V is used to quantitatively determine the percentage of cells within a population that are actively undergoing apoptosis. Apoptosis is characterized by a variety of morphological features such as loss of membrane asymmetry and attachment, condensation of the cytoplasm and nucleus, and internucleosomal cleavage of DNA. One of the earliest indications of apoptosis is the translocation of the membrane phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. Once exposed to the extracellular environment, binding sites on PS become available for Annexin V, a 35-36 kDa, Ca 2+-dependent, phospholipid binding protein with a high affinity for PS. The translocation of PS precedes other apoptotic processes such as loss of plasma membrane integrity, DNA fragmentation, and chromatin condensation. As such, Annexin V can be conjugated to biotin or to a fluorochrome such as FITC, PE, APC, Cy5, or Cy5.5, and used for the easy, flow cytometric identification of cells in the early stages of apoptosis. Because PS translocation also occurs during necrosis, Annexin V is not an absolute marker of apoptosis. Therefore, it is often used in conjunction with vital dyes such as 7-amino-actinomysin (7-AAD) or propidium iodide (PI), which bind to nucleic acids, but can only penetrate the plasma membrane when membrane integrity is breached, as occurs in the later stages of apoptosis or in necrosis.



Schematic representation of the Annexin V assay.

No Apoptosis = Cell Viability

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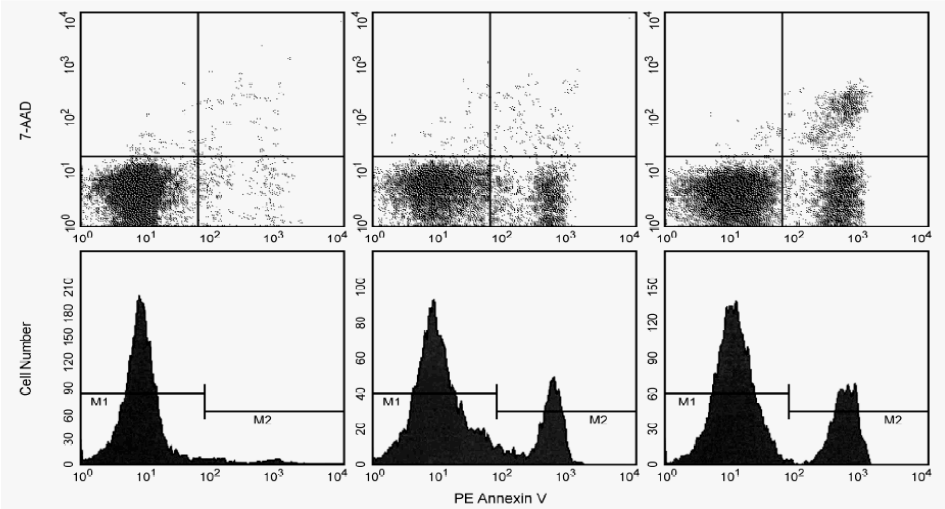
Cells that are negative for both Annexin V and the vital dye have no indications of apoptosis: PS translocation has not occurred and the plasma membrane is still intact.

Early Apoptosis

Cells that are Annexin V-positive and vital dye-negative, however, are in early apoptosis as PS translocation has occurred, yet the plasma membrane is still intact.

Late Apoptosis or Cell Death

Cells that are positive for both Annexin V and the vital dye are either in the late stages of apoptosis or are already dead, as PS translocation has occurred and the loss of plasma membrane integrity is observed.



Cells were untreated (upper left & lower left panels), treated for 5 hours (upper middle & lower middle panels) or 12 hours (upper right & lower right panels). Cells were incubated with PE Annexin V in a buffer containing 7-amino-actinomycin (7AAD) and analyzed by flow cytometry. Untreated cells were primarily PE Annexin V and 7-AAD negative, indicating that they were viable and undergoing apoptosis. After a 5 hour treatment there were two populations of cells; cells undergoing apoptosis (PE Annexin positive and 7-AAD negative), and cells that were viable but not undergoing apoptosis (PE Annexin V and 7-AAD negative).After a 12 hour treatment three populations of cells were identified; Cells that had die dor were in latest stage of apoptosis (PE Annexin V and 7-AAD positive), cells undergoing apoptosis (PE Annexin V positive and 7-AAD negative), and cells that were viable and not undergoing apoptosis (PE Annexin V and 7-AAD negative)

5. Safety precautions

Treed every sample containing human material such as AB serum and the lymphocyte samples as infectious material. Wear disposable gloves.

Sodium azide T+, N

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6. Reagents

6.1 *Chemicals*

Reagents	Formula	Supplier	order number	Store at (°C)
L-glutamine	-	Invitrogen	25030-024	-20
Penicillin-Streptomycin	-	Invitrogen	15140-122	-20
Foetal Bovine Serum	-	Invitrogen	10270-106	-20
PE Annexin V	-	BD Biosciences	556422	+4
7-AAD	-	BD Biosciences	559925	+4
Sodium azide	NaN3	Sigma	S-2002	RT
Annexin V binding buffer (10x)	-	BD Biosciences	556454	+4

6.2 *Facs Buffer (PBS with 2% FCS and 0.1% Sodium Azide)*

- 980 ml PBS
- 20 ml FBS
- 1 gram Sodium Azide

Exp.Date : 3 months at +4°C

7. Equipment and Accessories tools

7.1 *Equipment*

- Centiguge Hettich Rotanta 46 (UMC# 99-000-2142)
- Easypet pipet (Eppendorf, Germany, 4006173)
- Pipets 10-1000 µl (Gilson, The Hague, The Netherlands)
- Multistep pipet (Eppendorf, Germany)

7.2 *Accessories*

- 50 ml sterile polypropylene conical tubes (Falcon/ Becton Dickinson, Erembodegem, Belgium, 352070)
- Sterile 10 ml syringe (Becton Dickinson, Erembodegem, Belgium)
- 2 ml sterile disposable pipet (Falcon/ Becton Dickinson, Erembodegem, Belgium, 357507)
- 5 ml sterile disposable pipet (Falcon/ Becton Dickinson, Erembodegem, Belgium, 357543)
- 10 ml sterile disposable pipet (Falcon/ Becton Dickinson, Erembodegem, Belgium, 357551)
- 25 ml sterile disposable pipet (Falcon/ Becton Dickinson, Erembodegem, Belgium, 357525)
- Disposable gloves (Kimberly Clark, Zaventem, Belgium)

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8. Samples

8.1 Sample Collection

The amount of cells frozen and other data concerning a sample can be found in the cell storage

8.2 Sample Processing

All handlings of the sample should be done in a biohazard safety cabinet

9. Procedure

1 Wash cells twice with cold FACS buffer and then resuspend cells in 1x binding buffer (dilute 1 part binding buffer to 9 parts distilled H2O) at a concentration of 2x10E5 cells/well
2 Add 5 µl Annexin V PE and 5 µl of 7-AAD
3 Gently vortex the cells and incubate for 15 minutes at RT in the dark
4 Transfer the conditions to facs-tubes
5 Add 400 µl of 1x binding-buffer to each tube
6 Analyse by flow cytometry within 1 hr

Suggested controls for setting up flow cytometry

The following controls are used to set up compensation and quadrants:

1. Unstained cells
2. Cells stained with Annexin V PE alone
3. Cells stained with 7-AAD alone
4. Cells stained with Annexin V PE and 7-AAD
5. *DMSO treated cells stained with Annexin V PE and 7-AAD*

Induction of apoptosis by DMSO

The following protocol is used to test the Annexin V staining:

1. Add DMSO (final concentration 10 % (v/v)) to the cells
2. Incubate the cells for 1 hour at 37°C
3. Proceed with the Annexin V staining protocol to measure apoptosis

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