Instructions for use

ROTITEST® Annexin V

ROTITEST® Bio Analysis
For detection and enumeration of apoptotic cells ans simultaneous differentiation from necrosis

A. Introduction

Apoptosis is known to play an important role in maintaining the homeostasis and developmental processes in both plants and animals.
As of today, various indicators have been established such as caspase activity variation, DNA fragmentation and the so called phosphatidylserine (PS)-flip.
During the first stages of apoptosis, PS is translocated from the inner membran layer to the outer surface of the cell. The ROTITEST®-Annexin V Kit uses the Ca^{2+} dependent binding efficiency of Annexin V to PS in order to label cells with damaged cell membranes.
Additionally, propidium iodide is used to counter stain nuclei of cells with opened membranes, hence those which undergo necrotic degradation.
Thus, cells with yellow-green membrane staining only can be identified as apoptotic, while double stained cells (with yellow-green membranes plus orange-red nuclei) are classified as necrotic.

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B. Kit Contents

Sufficient for analysis of 50 samples of 10^5 cells each

- Annexin V-FITC Conjugate for 50 assays*
- PI Solution for 50 assays*
- Annexin V Binding Buffer for 50 assays*

* Corresponding to a cell concentration of 1 x 10^6 cells / ml.

C. Precaution

Reagents of this Kit should be handled with gloves only. Both FITC-labeled Annexin V and PI are light sensitive. All staining procedures must be performed without direct exposure to intense light.

D. Application

D.1 Prior to use

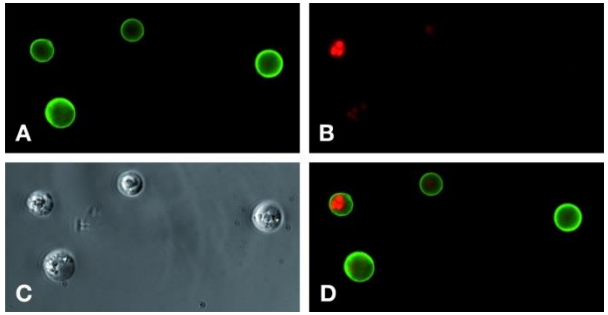
1. Dilute Annexin V Binding Buffer by 10-fold with distilled water.
2. Count your cells and calculate the amount of solution needed in order to achieve a concentration of 10^6 cells/ml.

D.2 Annexin V Staining of Suspension Cells

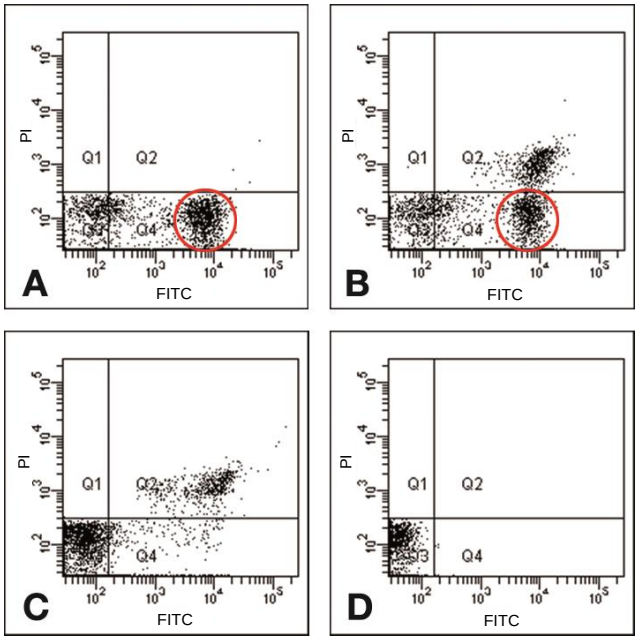
1. Centrifuge the cell suspension at 1.000 rpm for 3 minutes and remove supernatant.
2. Wash cells by addition of 1 prior vol. PBS.
3. Again, centrifuge at 1.000 rpm for 3 minutes and then remove supernatant.
4. Repeat steps 2 and 3 one more time.
5. Add 10-fold diluted Annexin V Binding Buffer to a final cell concentration of 1 x 10^6 cells / ml.
6. Transfer 100 µl of this cell suspension to a new tube.
7. Add 5 µl of Annexin V-FITC Conjugate, mix by slow pipetting.
8. Add 5 µl of PI solution, mix by slow pipetting.
9. Incubate 15 minutes at room temperature protected from light.
10. Add 400 µl of 10-fold diluted Annexin V Binding Buffer.
11. Apply the solution prepared in step 10 to flow cytometric assay or microscopic assay.

D.3 Annexin V Staining of Adherent Cells

1. Discard supernatant on the petri dish or plate.
2. Wash cells by addition of 1 prior vol. PBS.
3. Again, remove supernatant.
4. Repeat steps 2 and 3 one more time.
5. Detach the cells with Trypsin or EDTA
6. Add an appropriate volume of culture medium or PBS and transfer the cell suspension to a tube.
7. Centrifuge at 1.000 rpm for 3 minutes and then remove supernatant
8. Wash cells by addition of 1 prior vol. PBS.
9. Again, centrifuge at 1.000 rpm for 3 minutes and then remove supernatant.
10. Repeat steps 8 and 9 one more time.
11. Add 10-fold diluted Annexin V Binding Buffer to a final cell concentration of 1 x 10^6 cells / ml.
12. Transfer 100 µl of this cell suspension to a new tube.
13. Add 5 µl of Annexin V-FITC Conjugate, mix by slow pipetting.
14. Add 5 µl of PI solution, mix by slow pipetting.
15. Incubate 15 minutes at room temperature protected from light.
16. Add 400 µl of 10-fold diluted Annexin V Binding Buffer.
17. Apply the solution prepared in step 10 to flow cytometric assay or microscopic assay.



Fluorescence microscopic analysis of Jurkat cells. Assayed using 10^5 cells in 100 µl Annexin binding buffer, 5 µl Annexin V-FITC conjugate and 5 µl propidium iodide solution, 15 min. RT.
A) FITC fluorescence (green), B) propidium iodide fluorescence (red), C) bright field image, D) composite image. Apoptotic cells are FITC positive, PI negative. FITC pos/PI pos cells may be classified as being necrotic. Healthy cells are FITC/PI neg.



Flow cytometry analysis of Jurkat cells. Assayed using $5x10^5$ cells in 500 µl Annexin binding buffer, 25 µl Annexin V-FITC conjugate and 25 µl propidium iodide solution, 15 min. RT.
FITC: FITC staining (binding of Annexin-FITC conjugate), PI: Propidium iodide staining
A,B) Cells after induction of apoptosis by Staurosporin, under pure annexin-FITC staining (A) or under annexin-FITC / PI staining (B), respectively. C,D) Uninduced control cells under annexin-FITC / PI staining (C) and without staining (D), respectively .
Due to the binding of Annexin V to PS on the outer membrane surface, apoptotic cells are Annexin-FITC positive, but PI negative (Q4, circle). Q3 displays healthy cells (neg/neg), Q2 necrotic cells (Annexin-FITC pos and PI pos). Cells in Q1 (PI pos/ Annexin-FITC neg) are dead, non-apoptotic cells.

F. Tips and Tricks, Important Notes:

Since cell detachment process may cause specific membrane damage, Annexin V-FITC flow cytometric analysis is not frequently performed with adherent cells. However, Casiola-Rosen *et al*.[1)] and van Engelend *et al.*[2)] reported methods on utilizing Annexin V for flow cytometry with adherent cell types. For protocols, please see there.

G. Material and Equipment Additionally Required

- Flow cytometer or fluorescence microscope
  Excitation / Emission:
  Annexin V-FITC: 494 nm / 518 nm;
  PI: 535 nm / 617 nm
- Phosphate buffered saline (PBS, Roti®Stock 10x PBS Art. No. 1058.1)
- EDTA (Roti®Cell PBS/EDTA, Art. No. 9152.1, Roti®Cell DPBS/EDTA, Art. No. 9135.1)
- Trypsin (Art. No. 2193.1)
- Deionized water

B. Storage

Do not freeze. Store the kit at 0-5 °C and protect it from long exposure to light.

H. References

[1)] Casciola-Rosen L. *et al.* (1996) *Proc Natl Acad Sci USA*, 93 :1624.
[2)] van Engeland M. *et al.* (1996) *Cytometry*, 24:131.

ROTITEST® Annexin V

7735.1 50 Assays