

Method for Analysing Apoptotic Cells via Annexin V Binding

Background

This method is used for the detection of apoptotic cells via Annexin V binding to phosphatidyl serine (PS) at cell surface of apoptotic cells.

Apoptosis is characterized by morphological and biochemical features that occur at different stages. One of these is the exposure of PS on the outer leaflets of the plasma membrane. Annexin V preferentially binds to negatively charged phospholipids like PS in the presence of Ca²⁺ and therefore can be used in the detection of apoptotic cells.

This technique is not ideal for cells isolated from solid tissue or adherent cells because of the damage to plasma membrane caused in the attempt to disaggregate cells to have a single cell suspension. The binding of Annexin V to PS can be affected particularly in adherent cells, which are usually detached from plastic dishes by enzymatic, chemical, or mechanical treatment. Any procedure/process/phenomenon (scraping/trypsinisation/necrosis) that affects the integrity of the plasma membrane will result in cell becoming positive for Annexin V. PI staining provides evidence of the loss of plasma membrane integrity. However the damage to cell membrane can be minimised via careful sample preparation. *Care should be taken in preparatory procedures.*

Materials

1. **Any fluorochrome conjugated Annexin V** (i.e. FITC, Alexa 488, APC , Alexa 647, etc)
2. **1x PBS**
3. **Binding buffer:**

HEPES buffer: 10 mM HEPES/NaOH, (pH 7.4), 150 mM NaCl, 5 mM KCl, 5 mM MgCl₂, and 1.8 mM CaCl₂ (**can be bought pre-made from BD (cat. No: 556454)**)

4. **Viability dyes**, i.e. PI, DAPI, 7AAD , etc
5. **Cells** in suspension (usually 1 million per sample). When working with adherent cells, be sure to harvest all cells including floating cells in the supernatant (apoptotic cells become more buoyant)

Additional Considerations

1. **Single colour stained control:** when combining fluorochromes with overlapping fluorescence emission, a single colour positive control should be prepared for each dye in the experiment.
2. **Negative control:** unstained cells can be used to set the base line of each parameter.

Equipment

1. **Centrifuge.**
2. **Pipettes.** You will need two: one in the range of 10-100 μ l, and another ranging from 100-1000 μ l.
3. **12x75 mm polystyrene/polypropylene tubes.** Depending on which machine you wish to use (LSR II prefers polystyrene while the CyAn prefers polypropylene).
4. **Ice bucket with cover.** Generally, cells are more stable and tolerate stress better when they're cold. The cover keeps samples in the dark and prevents light from bleaching the fluorochromes.
5. **Flow cytometer.** We have a variety of machines at your disposition including a BD LSR II, BD FACSAArray and a Beckman Coulter CyAn. Each machine has different capabilities, strengths and weakness so be sure to check with us which machine is suited for your needs.

Procedure

1. Harvest your cells and, if you can, adjust the number of cells to roughly 1 million per sample.
2. Dilute the Annexin V conjugate 1 in 100 in Annexin V binding buffer. (if staining more than one sample, prepare a batch solution for all samples and mix well).
3. Resuspend each pellet in 500 μ l Annexin V conjugate/binding buffer mixture.
4. Keep the samples at RT in the dark for around 20 minutes (30 or more minutes on ice), cover the ice bucket.
5. Add to each sample 50-100 μ l of 50 μ g/ml PI solution (or 5 μ g per sample of any other viability dye) prior to analysis. Transfer sample onto ice.

Keep the cells on ice and covered, until your scheduled time on the flow cytometer. Remember apoptosis is an ongoing process so keep your cells on ice at all time. Annexin V stained cells should not be kept for prolonged times before measurement.