Annexin V staining of adherent cells for flow cytometry

Materials and Reagents

Full Name	Short Name	Catalog Number
Microwell plates (round-bottom wells) or tubes (12 x 75-mm polypropylene round-bottom test tubes)		N/A
1X PBS Buffer	PBS	554781
Accutase™ Cell Detachment Solution	Accutase	561527
Annexin V conjugates	Annexin V	*
Propidium Iodide staining solution or	PI	556463
BD Via-Probe™ Cell Viability Solution	7-AAD	555816
Annexin V Binding Buffer, 10X concentrate	Annexin V Binding Buffer	556454

N/A, not applicable
*Select at www.bdbiosciences.com

Procedural Notes

- For optimal results, we recommend titration or reagents and conditions for inducing apoptosis.
- Optional: cells may be stained with antibodies against cell surface markers and washed before proceeding with Annexin V staining. Investigators might want to check the effects of the staining procedure on changes in cell surface levels of Annexin V, for example, by inducing control tubes with or without cell surface antibodies.
- Methods for utilizing Annexin V binding on adherent cells (ie, monolayer) have also been described by van Engeland et al and Casciola-Rosen et al. However, these methods are not performed as a routine quality control for the Annexin V conjugates. During development, the following procedure has been used successfully for NIH 3T3 and E14 mouse ES cells, but not for 293 or HeLa cells for which Annexin V^+ 7-AAD^- and Annexin V^- 7-AAD^- cells were not clearly resolved. Individual laboratories should verify the use of other adherent cell lines or methods of apoptosis induction.
- Use PI with FITC-, APC-, or BD Horizon™ V450-conjugated Annexin V; use 7-AAD with PE-, Cy™5-, Cy™5.5-, or BD Horizon™ V500-conjugated Annexin V
- We suggest these controls for flow cytometric analysis of Annexin V samples.
    1. Unstained cells
    2. Cells stained with Annexin V conjugate alone (no PI, no 7-AAD)
    3. Cells stained with PI alone (no Annexin V conjugate)

Procedure: Preparing PBS Buffer

1. Add 8 g of NaCl, 0.2 g of KCl, 1.44 g of $Na_2HPO_4 \bullet 7H_2O$, and 0.24 g of $KHPO_4$ to 1 liter of H_2O.
2. Adjust the pH to 7.2.
3. Autoclave and store at room temperature (RT).

Procedure: Staining

1. The day before induction of apoptosis, plate cells at a suitable confluency, for example, ~250,000 NIH 3T3 cells per well for a 6-well plate (3-mL volume per well) using standard cell culture medium.

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2. After ~18 hours, check the wells for floating (dead) cells and remove if necessary by pipet. Replace with new culture medium to the original volume.
3. Treat cells to induce apoptosis, for example, by adding camptothecin (5-20 µM) and incubate for 24 hours for NIH 3T3 cells or 6 hours for E14 cells.
4. Collect cell culture medium into 15-mL tubes.
5. Add Accutase to each well, enough to cover the surface (ie, 1 mL for each well of a 6-well plate) and incubate for 1-2 minutes at RT. (Some cell lines may require longer incubation, for example, 10 minutes).
6. If needed, gently tap the side of the plate or flask to help detach the cells from the surface.
7. Add 2 mL of medium to each well and transfer the contents (~3 mL) to the 15-mL tubes.
8. Centrifuge and discard the supernatant.
9. Optional: cells may be stained with antibodies against cell surface antigens prior to the Annexin V staining procedure. If you are not staining cells, proceed to step 10.
    a) Wash cells with Stain Buffer and resuspend in 100 µL (in 12 x 75-mm tubes or 96-well plates).
    b) Add antibodies against cell surface antigens and incubate for 20-45 minutes in the dark (either on ice or at RT).
    c) Wash cells twice (centrifuging at 300*g*) with Stain Buffer (100-200 µL for 96-well plates or 1-2 mL for tubes).
10. Wash cells twice with cold PBS and then resuspend cells in 1X Binding Buffer at a concentration of ~1 x 10^6 cells/mL.
11. Transfer 100 µL of the solution (~1 x 10^5 cells) to a 5-mL culture tube (or 12 x 75-mm tube).
12. Add Annexin V and either PI or 7-AAD (see procedural notes) as described in the Technical Data Sheet or the Annexin V apoptosis kit manual.
13. Gently mix the cells and incubate for 15 minutes at RT in the dark.
14. Add 400 µL of 1X Binding Buffer to each tube. Analyze by flow cytometry as soon as possible (within 1 hour).

References

van Engeland M, Ramaekers FC, Schutte B, Reutelingsperger CPM. A novel assay to measure loss of plasma membrane asymmetry during apoptosis of adherent cells in culture. *Cytometry*. 1996; 24:131-139.

Casciola-Rosen L, Rosen A, Petri M, Schlissel M. Surface blebs on apoptotic cells are sites of enhanced procoagulant activity: implications for coagulation events and antigenic spread in systemic lupus erythematosus*. Proc Nat Acad Sci USA.* 1996; 93:1624-1629.

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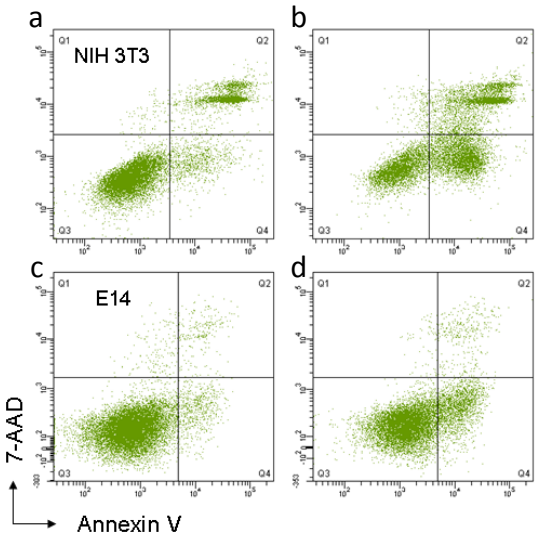

Figure 1. NIH 3T3 cells were either untreated (a) or treated with 20µM camptothecin for 24 hours (b). E14 cells were either untreated (c) or treated with camptothecin for 6 hours (d). Cells were harvested using Accutase cell detachment solution (Cat. No. 561527) and apoptosis measured using the PE Annexin V Apoptosis Detection Kit (Cat. No. 559763).

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