

# Multiparametric Detection of Apoptosis (2)

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## **Background**

This method is used for detection of apoptotic cells via five different morphological and biochemical changes in the cell: loss of cell membrane asymmetry by phosphatidylserine externalisation, loss of cell membrane integrity, loss of mitochondrial membrane potential, chromatin condensation, and degradation of DNA. It's a combination of Annexin V and 7AAD staining, the detection of mitochondria membrane potential changes using TMRE, the analysis of chromatin condensation based on Hoechst red shift, and DNA degradation based on subG1 analysis, all together in the same sample tube.

Furthermore the use of Hoechst33342 allows examination of cell cycle of live and dying cells.

## **Materials**

1. **TMRE** (40 $\mu$ M in PBS)
2. **Annexin V-AlexaFluor647 conjugate**
3. **Annexin V Binding buffer:** HEPES buffer: 10mM HEPES/NaOH, (pH 7.4), 150mM NaCl, 5mM KCl, 5 mM MgCl<sub>2</sub>, and 1.8mM CaCl<sub>2</sub> (**can be bought pre-made from BD, cat. No: 556454**)
4. **1x PBS**
5. **7AAD solution** (25 $\mu$ g/ml)
6. **Propidium Iodide solution** (50 $\mu$ g/ml in PBS)
7. **70% ethanol** (in DI water)
8. **RNase A** (100 $\mu$ g/ml)
9. **SubG1 Phosphate-Citrate buffer:** 0.2M Na<sub>2</sub>HPO<sub>4</sub> (disodium phosphate, 2.84g /100ml) 0.1M Citric Acid (2.10g /100ml). Mix 192 parts 0.2M Na<sub>2</sub>HPO<sub>4</sub> and 8 parts 0.1M citric acid and adjust to pH 7.8.
10. **Hoechst33342:** 5-20 $\mu$ g/ml (requires optimisation).

## **Equipment**

1. **Centrifuge.**
2. **Pipettes.** You will need one in the range of 2-10 $\mu$ l, one in the range of 10-100 $\mu$ l, and another ranging from 100-1000 $\mu$ 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 insult better when they're cold. The cover keeps light out, which could bleach the fluorochromes.
5. **Flow cytometer.** We have a variety of machines at your disposal including a BD LSR II, BD FACSAria and a Beckman Coulter CyAn. Each machine has different capabilities, strengths and weaknesses so be sure to check with us which machine is suited for your needs.

### **Additional Considerations**

1. **Single colour 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.

### **Procedure**

1. Harvest cells in the appropriate manner and wash in PBS.
2. Resuspend one million cells in 1ml culture cell medium. Add Hoechst33342 to cells and incubate for 50 minutes at 37°C in the dark.
3. Add TMRE at 40nM and further incubate cells at 37°C in the dark for 10 minutes.
4. Centrifuge. Use a pipette to remove the liquid or decant supernatant. Be careful not to lose the pellet. A slight amount of liquid can remain (usually you will see the cell pellet at the bottom).
5. Resuspend cell in PBS and repeat step 4.
6. Dilute the Annexin V conjugate 1 in 100 in Annexin V binding buffer plus Hoechst33342 (if staining more than one sample, prepare a batch solution for all samples and mix well).
7. Resuspend pellet in 500µl Annexin V conjugate/binding buffer/Hoechst33342 mixture incubate for 20 minutes at room temperature in the dark.
8. Transfer cells onto ice and add 50µl of 7AAD solution.
9. Incubate for 15 minutes.
10. Run the samples on LSR II. Use Hoechst blue (450 off UV) Height vs. Area to get rid of doublets and clumps. Use 620LP off UV for Hoechst Red fluorescence. Probe for Annexin V binding (660 off Red laser), and TMRE (580 off Blue laser) and 7AAD (670 off Blue laser) staining. Save 10 to 30,000 cells.
11. Centrifuge at 400 g for 5 minutes. Use a pipette to remove the liquid. Be careful not to disturb the pellet.
12. Fix in cold 70% ethanol for at least 30 minutes on ice. Add drop wise to the cell pellet while vortexing. This should ensure fixation of all cells and minimise clumping. Cells can be kept in ethanol overnight or longer.
13. Wash twice in 1ml Sub G1-Phosphate-citrate buffer. Spin down and be careful to avoid cell loss when discarding supernatant especially after spinning out of ethanol.
14. Add 50µl of RNase A to the pellet.
15. Add 200µl propidium iodide.
16. Analyse by flow cytometry for subG1 accumulation of late apoptotic cells.

### **Cautions:**

TMRE emits at around 580 nm. For subG1 analysis, use 670 filter for PI detection to avoid any residual TMRE staining.

When analysing samples for Hoechst or subG1, be sure to collect Hoechst and PI in linear scale. Use a dot plot showing Area vs. Height or Width to gate out doublets and clumps and analyse at a low flow rate under 400 events/second.