SubG1 Analysis Using Propidium Iodide

Background

This is a method used to detect cells that have lost some of their DNA in late stage of apoptosis process following endonucleases activity. Endonucleases degrade DNA in small fragments of about 180 bp, which accumulate in the cell. After ethanol fixation and wash with a phosphate-citrate buffer, these DNA oligimeres leak out of the cell decreasing the DNA content. Using the nucleic acid stain propidium iodide (PI), the number of hypodiploid cells undergoing this process can be counted in subG1 region of PI histogram.

Materials

1. 70% ethanol (in DI water)
2. 1x PBS
3. PI solution in PBS (50µg/ml)
4. RNase A solution in PBS (100µg/ml)
5. SubG1 Phosphate-Citrate buffer:
    - 0.2M Na_2HPO4 (disodium phosphate): 2.84g /100ml
    - 0.1M Citric acid: 2.10g /100ml*)*

    (Mix 192 parts 0.2M Na2HPO4 and 8 parts 0.1M citric acid and adjust to pH 7.8)

Equipment

1. **Centrifuge.**
2. **Pipettes.** You will need one in the range of 2-10µl, one in the range of 10-100µl, and another ranging from 100-1000µl.
3. **Vortex mixer.** You *could* mix by tapping or shaking the tubes, but a mixer will give much more reproducible results in most cases.
4. **12x75 mm polystyrene/polypropylene tubes.** Depending on which machine you wish to use (LSR II prefers polystyrene while the CyAn prefers polypropylene.
5. **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.
6. **Flow cytometer.** We have a variety of machines at you disposable including a BD LSR II, BD FACSArray 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 cells in the appropriate manner and wash in PBS. If using adherent cells be sure to harvest all cells including the supernatant (apoptotic cells become more buoyant). This protocol is optimal for 1 million cells per sample.
2. Fix in 1ml cold 70% ethanol. Add drop wise to cell pellet while vortexing. This should ensure fixation of all cells and minimise clumping.
3. Fix for at least 30 minutes on ice. Specimens can be left at this stage for several weeks (make sure you seal the tubes for long term storage).

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4. Pellet cells at higher speed (compared to live cells) for 5 minutes; decant supernatant being careful not to disturb the pellet. Note that ethanol-fixed cells require higher centrifugal speeds to be pelleted compared to unfixed cells since they become more buoyant upon fixation.
5. Loosen the pellet each time before adding wash buffer. Wash twice in Phosphate-citrate buffer at RT. Centrifugal speed may need to be adjusted depending on the cells. DO NOT USE BRAKE when spinning as apoptotic cells become buoyant.
6. To ensure that only DNA is stained, treat cells with 50µl Ribonuclease A.
7. Add 450µl propidium iodide per 1 million cells directly to cells in RNase A solution.
8. Incubate for 10 to 15 minutes (for most cells this is enough time, however certain cells i.e. fibroblast, require 24 hours incubation at 4°C to stain properly.
9. Analyse samples by flow cytometry in PI/RNaseA solution (no need to wash cells). Save at least 10,000 single cells.

Flow analysis:

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

Cautions:

SubG1 gate underestimates the number of apoptotic cells as cells can dye from all cell cycle phases and hypodiploid cells can temporarily accumulate above G1 phase. DNA content histogram obtained by this method can only be used to estimate subG1 cells and cannot be used to estimate proportions of cells in all cell cycle phases when a sample shows an accumulation of apoptotic cells in subG1.

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