Cell cycle staining with Propidium Iodide for the cytoplasmic GFP transfected cells using Paraformaldehyde and Ethanol

1. Test principle

For optimal PI staining, cells require permeabilization with ethanol to allow the dye access to the nucleus. However, such treatment with ethanol results in the loss of GFP fluorescence as the soluble, cytoplasmic GFP leaks out of the cells following permeabilization. In contrast, the use of fixatives (e.g. paraformaldehyde) that retain GFP in the cells leads to DNA histograms with unacceptably high codfficients of variations (CV) for the G1/G0 peaks.
This protocol is for cell fixation/ permeabilization for combined measurement of GFP expression and PI DNA content. Cells are fixed with 1% formaldehyde followed by treatment with 70% ethanol for cell membrane solubilization, and then stained with PI in the presence of RNAse A for DNA content.

2. Specimen

GFP transfected cells

3. Materials and reagents

- 5ml Falcon polypropylene Round-Bottom test tubes (12x75 mm)
- cooling centrifuge
- 37°C water bath
- 40 μm nylon mesh (BD Falcon Cell Strainer # 352340) or 70 μm nylon mesh
- sterile-filtered Phosphate-buffered saline (PBS), 4°C
- **Fixation Solution** (4°C): Add 2g high purity paraformaldehyde (electron microscopy grade from Polysciences, 2% w/v final) to 100 ml PBS. Heat to 70°C in a fume hood until the paraformaldehyde completely dissolves (~1hr). Cool to room temperature and adjust pH to 7.2. Store at 2°C to 8°C protected from light. The solution is stable for at least 1 month. Check pH periodically.
- **70% ethanol**, -20°C
- **Propidium iodide (PI) solution**:
  Stock solution: Dissolve 1 mg PI (Sigma, P 4170) in 1 ml dH_2O at 1 mg/ml.
  Store aliquots at -20°C for up to 2 years. Aliquots that are frequently used can be stored at 4°C protected from light for up to 2 months. Discard solutions of PI that have been exposed to room temperature for more than 48 hr, or if they appear dark red. Keep it in the dark.
  Working solution: Dilute PI stock solution 1:25 with PBS. Final concentration of PI is 40 μg/ml.
- **RNAse A solution:**
  Dissolve 50 mg RNAse A (Sigma, R-4875) in 50 ml of PBS and adjust to 0.1% Tween-20.
  Place solution into a 95°C water bath for 30 min.
  Allow the solution to cool on ice for 1 hr. Remove precipitate by filtering through a 0.2 μm filter.
  Solution can be stored for up to 6 months at 4°C or in aliquots be frozen at -20°C.

4. Controls

- Cells transfected with GFP alone without PI added
- No GFP transfected and no drug treated cells stained with PI

5. Procedure

a) Count cells

b) Place 1x 10^6 cells in to a 12x75 mm test tube and wash once with PBS by centrifuging 5 min at 300 x g, 4°C.

c) Fix cells with 1% paraformaldehyde: Remove supernatant by aspiration and add 500 µl cold PBS to the cell pellet. Mix gently. Add 500 µl cold (4°C) 2% fixation solution and mix again. Incubate 1 hr at 4°C.
   - *Different concetrations of formadldehyde fixative may be needed for optimal retention of GFP in various cell types, and for obtaining acceptable CV on DNA histograms.*

d) Permeabilize cells with ethanol: Centrifuge cells 5 min at 300 x g, 4°C. Remove supernatant by aspiration and wash once with cold 3 ml PBS. Add 1 ml 70% ethanol at -20°C drop-wise to the cell pellet with the tube sitting on a vortex mixer. Incubate cell suspension overnight at 4°C.
   - *Be careful for aspiration of the supernatant since a cell pellet may not be visible after the fixation steip with formaldehyde. Fixed cells aggregate less well and tend to spread out at the bottom of the tube.*
   - *Vortexing cells gently during the addition of ethanol can reduce the formation of cell clumps, but too vigorous vortexing can lead to cell disruption.*
   - *Incubation times with 70% ethanol should not be less than 2 hrs.*

e) Stain cells with PI: Centrifuge cells 5 min at 300 x g, 4°C. Remove supernatant, wash once with 3ml PBS, and add 1ml propidium iodide (PI) working solution. Incubate cell suspension 30 min in a 37°C water bath in the dark. If needed, filter samples through a 40 or 70 um nylon mesh to remove clumps.

f) Analyze samples on the flow cytometer.

Note: PI is a nucleic acid-specific, red-fluorescent dye. It is also a suspected carcinogen, so employ appropriate safety precautions when working with this dye.

Reference: Current Protocols in Cytometry (2001) 7.16.1, Ingrid Schmid and Kathleen M. Sakamoto