

## Propidium Iodide Staining for DNA Content in Ethanol Fixed Cells

The most frequently used method for measuring DNA content of cells involves the fixation and permeabilization of the cells with ethanol. The ethanol treatment provides excellent access of the dye to allow the intercalation of the dye into the DNA grooves. Formaldehyde treatment of the cells, while adequately fixing and permeabilizing the cell interferes with access of the dye to the DNA. This is apparently due to the fixation of chromatin proteins onto the DNA leading to steric hindrance of the dye access to the DNA grooves.

The cells should be prepared into a single cell suspension. For adherent cell lines the cells will need to be released from the culture dish. Trypsin with or without EDTA is usually sufficient but there are other methods. Scraping cells off culture surfaces is **NOT** recommended. Solid tissues will need to be disaggregated using enzyme treatment usually with collagenase and proteinases. After the cells are prepared into a single cell suspension they should be washed with PBS and  $1-2 \times 10^6$  cells placed in 5ml of PBS. Note: Centrifugations should be done in a regular centrifuge with a swing bucket rotor. Do NOT use micro centrifuges and Eppendorf tubes.

1. Prepare the ethanol (70%) and bring to 4°C or colder (place in a -20°C freezer for several hours). Place 4.5ml into each polypropylene centrifuge tube. Prepare one tube of ethanol for each 5ml of cell suspension. Keep ethanol on ice at all times.
2. Prepare the staining solution. To 10ml of 0.1% TritonX-100 in PBS add 2mg DNase-free RNase and 200ug of 1mg/ml propidium iodide in water. RNase may be purchased DNase free or made DNase free by placing a small volume in a 100°C water bath for 5 min. PI made at 1mg/ml and kept at 4°C and covered with foil is good for several months. Make diluted staining solution fresh each day.
3. Place 5ml of cell suspension in a polypropylene test tube (round bottom is best) and centrifuge to pellet the cells. Decant supernatant immediately after centrifugation stops.
4. Break up cell pellet and then add 0.5ml of PBS and completely resuspend the cells.
5. Add the cell suspension to one tube of ethanol and mix thoroughly.
6. Keep the cells in the ethanol for at least 2 hours. Cells in ethanol may be stored at -20°C for several months.
7. Centrifuge the ethanol suspension of cells and decant the ethanol completely. Touch the edge of the centrifuge tube to a paper towel. Do not use a Pasteur pipette to remove the ethanol.
8. Suspend the cells in 5ml of PBS and wait 1-2 minutes. Centrifuge cells. Some cell types do not like to centrifuge well and the pellet almost will disappear. It sometimes helps to include 0.5% BSA in the PBS.
9. Break up the cell pellet and then resuspend the cells in 1ml of the staining solution.
10. Keep at 37°C for 15 min. or at room temp for 30 min. Place on ice and keep covered with foil until analyzed on the flow cytometer. This can be overnight (some claim better results with overnight).

**Note:** For the best results all of the cells samples should be at the same concentration. Changing the cell concentration between samples will result in different intensities of staining. This is due to the fact that the binding is dictated by the law of thermodynamics which states that the binding is due to the concentration (not amount) of the reactants – in this case DNA and propidium iodide. If, after staining, cells ever need to be diluted be sure to dilute them using the staining solution or at least PBS with the same concentration of PI as the staining solution. Since the binding of PI to the DNA is an equilibrium reaction diluting the cell suspension with only PBS will change the DNA and PI concentrations and thus change the staining level of the DNA.

Internal standards can be included with the cells and are especially useful in situations where cells with different DNA content could be present – e.g. in clinical cancer samples where both diploid and hyperdiploid (aneuploid) cells could both be present. These are usually chicken RBCs or trout RBCs.

When analyzing the cells run the cytometer at a very low sample pressure to maximize precise alignment in the laser and thus the best CVs. Be sure to include doublet discrimination (PI area vs PI height) as part of the analysis strategy.