

Hoechst33342 and Pyronin Y Staining for G0/G1 Separation

Background

This is a method for the separation of G0 and G1 cell cycle phases. Hoechst is an exclusive DNA dye while Pyronin Y reacts with both DNA and RNA. In the presence of Hoechst, Pyronin Y reaction with DNA is blocked, and Pyronin Y stains RNA only. When cells are stained first with Hoechst33342 and then with Pyronin Y it is possible to distinguish DNA from RNA. Furthermore, quiescent cells, which are arrested in G0 phase, have lower level of RNA compared to active cells (G1 phase). This method is suitable for targeting senescent and resting cells.

Materials

1. Hoechst33342 1mg/ml (Sigma)
2. Pyronin Y 100µg/ml (Sigma)

Additional Considerations

1. **Single colour controls:** stain one sample with Pyronin Y only and one with Hoechst33342 only.
2. **Negative Sample:** An amount of unstained cells/sample used to initially adjust voltages on machine.

Equipment

1. **Centrifuge.**
2. **Pipettes.** You will need two: 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.** You need an instrument that has a true UV laser (350 nm excitation wavelength). We have BD LSRII (analysis only) and BC MoFlo XDP (analysis and cell sorting) that you could use to perform this assay.

Procedure

1. Harvest cells in the appropriate manner.
2. Resuspend cells in 1ml cell culture medium containing 10µg/ml Hoechst33342.

3. Incubate at 37°C for 45 minutes (Hoechst concentration and incubation time may require optimisation).
4. Add directly to the cells 5µl of 100µg/ml Pyronin Y.
5. Incubate at 37°C for a further 15 minutes.
6. Transfer cells onto ice.
7. Analyse samples by flow cytometry (no need to wash cells).

Flow analysis:

Keep the cells covered on ice until your scheduled time on the flow cytometer.

When analysing samples, be sure to collect both Hoechst and Pyronin Y in linear scale. Hoechst is efficiently excited by 350nm UV laser and emits at 450 nm. Pyronin Y is excited by the 488 laser and emits at 575 nm. This means in our facility you need to use LSRII (analysis only) or MoFlo XDP (analysis and cell sorting), which are fitted with true 350nm UV lasers. Use a dot plot showing Hoechst parameters Area vs Height or Area vs Width to gate out doublets and clumps and analyse at a low flow rate less than 400 events/second. A dot plot with Hoechst in X-axis and Pyronin Y in Y-axis should show a distribution of DNA and RNA content with G0 cells having lower Pyronin Y signal. A good resolution of cell cycle phases is crucial for the success of this assay. Hoechst concentration should be titrated and incubation time should be optimised for each type of cells to achieve a good DNA staining and hence good resolution of cell cycle phases.