

# Mitotic Cells and Cell Cycle

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## **Background**

This is a method to detect cells undergoing Mitosis alongside cell cycle. This method can also be used for detection of internal antigens of molecular weight higher than 50KDa and analysis of DNA content. This allows the user to correlate internal antigen expression and cell cycle.

This method can be performed with both direct and indirect staining. Here indirect staining is outlined. For smaller antigens see appropriate protocol that combines paraformaldehyde and ethanol fixation.

## **Materials**

1. **70% Ethanol**
2. **PBS + 0.1% Triton X**
3. **PBS + 0.1% Triton X + 3% BSA**
4. **Rabbit Anti Phospho Histone 3** (Upstate, cat: 06-570 (ser10))
5. **Anti-Rabbit conjugated antibody** (i.e. Alexa 647)
6. **PI** 50µg/ml
7. **RNase** 100µg/ml

## **Additional Considerations**

**Negative Sample:** An amount of unstained cells is used to initially adjust settings of the machine. No other controls are needed.

## **Equipment**

1. **Centrifuge.**
2. **Pipettes.** You will need three: one in the range of 2-10µl, one in the range of 10-100µl, and another one ranging from 100-1000µl.
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.

## **Procedure**

1. Harvest cells in the appropriate manner and wash in PBS.

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).
4. Pellet cells at higher speed for 5 minutes; decant the supernatant being careful not to lose the pellet. Note that ethanol-fixed cells require higher centrifugal speeds to pellet compared to unfixed cells since they become more buoyant upon fixation.
5. Wash once with PBS + 0.1% Triton X solution.
6. Wash once with PBS + 0.1% Triton X + 3% BSA.
7. Pour off supernatant and add directly to the pellet 2µl of rabbit Anti-Phospho Histone 3 antibody, mix well and incubate at room temperature for 45 minutes.
8. Wash twice with PBS + 0.1% Triton X solution.
9. Add directly to the pellet 5µl of AlexaFluor647 anti-rabbit secondary antibody, mix well and incubate at room temperature in the dark for 30 minutes.
10. Wash twice with PBS.
11. Add 50µl RNase A and 200µl PI and incubate for 5-10 minute at room temperature in the dark.
12. Analyse for cell cycle profile and Mitotic cells, saving at least 10,000 single cells.

#### Flow analysis:

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

When analysing samples, be sure to collect PI in 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. A dot plot showing PI in X-axis vs. AlexaFluor647 in Y-axis should show mitotic cells at the top of G2M phase.