

Cell Proliferation by Flow Cytometry (BrdU and PI)

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This method, modified from Hoy CA, et.al, is applied to adherent cell cultures. It may also work with suspension cultures, with modifications germane to their culture conditions.

Notes:

- An ideal sample is $1-2 \times 10^6$ cells. Keep all samples to about the same number of cells to avoid artifacts associated with cell concentration changes.
- Flow cytometry setup controls needed:
 - 1) Cells stained with PI alone.
 - 2) Cells with no BrdU pulse, and only the antibodies added.
- Amount of BrdU added may vary according to cell type, and proliferative potential.
- BrdU is light sensitive and should be added in the dark.
- Cells pulsed with BrdU may be photosensitive -- incubations should be in the dark as well.

Reagents (Product code)

BrdU (5-bromo-deoxyuridine) -- Sigma (B-5002)
RNase A (from bovine pancreas) -- Boehringer Mannheim (109-169)
Tween 20 -- Fisher Biotech (BP337-500)
Triton X-100 -- Sigma (X-100)
Propidium Iodide (PI) -- Sigma (P-4170)
Ca⁺⁺, Mg⁺⁺-free Delbecco's PBS
HPLC-grade H₂O
Fetal calf Serum (FCS)
Internal standard (IS, trout erythrocyte nuclei) – BioSure (1008)

Antibodies

Purified Mouse-anti-BrdU - Pharmingen (33281A)
Goat-anti-Mouse IgG (Whole molecule) FITC conjugate - Sigma (F0257)

Solutions

0.5 mg/ml RNase A in PBS
PTS -- PBS with 0.5 % Tween 20 and 5 % FCS
HCl-Triton -- 0.1 N HCl containing 0.7 % Triton X-100
PI stock (20 µg/ml in PBS)

Labeling Procedure

1. At desired time point(s), incubate cells with 30 μ M BrdU for 15 min.
2. Remove BrdU media. Rinse 1x with PBS.
3. Detach cells -- as appropriate; trypsin, EDTA, etc. -- and resuspend in 10 ml media.
4. Permeabilize cells:
Determine cell count. Aliquot desired number of cells per test sample to 15 ml conical tubes. Pellet at 1200 rpm for 5 min at 4°C. Decant supernatant, wash with 3 ml PBS, and re-pellet. Resuspend pellet in 0.3 ml PBS, agitate gently, then add 0.7ml ice-cold 100 % EtOH slowly. Mix gently with a 1 ml glass transfer pipette. The cell concentration following permeabilization should be approximately 10^6 cells/sample. Samples can be stored in EtOH for up to 2 weeks at 4°C.
5. Add IS if desired, at a concentration of 10^5 IS per 10^6 cells. Mix gently with a 1 ml glass transfer pipette. Pellet at 1100 rpm for 8 min at 4°C. Decant supernatant, wash with 5 ml PBS, re-pellet and decant supernatant.
6. Add 1 ml PBS containing 0.5 mg/ml RNase A. Agitate tube gently. Incubate at 37°C for 30 min. Add 5 ml PBS, pellet at 1100 rpm, 8 min at 4°C. Decant supernatant.
7. Agitate pellet, then resuspend with 1 ml of HCl-Triton solution. Vortex gently. Incubate on ice for 10 min. Add 5 ml PBS, pellet at 1100 rpm for 8 min at 4°C. Decant supernatant and blot gently to dry.
8. Add 1 ml sterile HPLC water, vortex gently. Incubate at 97°C for 15 min. Note: be sure to cap tubes loosely to allow for expansion, and keep lid on water bath to control heat range.
9. Immediately chill in ice-water bath for 15 min. Add 5 ml PBS containing 0.5 % Tween 20. Pellet at 1100 rpm, 8 min at 4°C. Decant supernatant.
10. Add 100 μ l PBT. Agitate gently with transfer pipette. Transfer sample to a 1.5 ml microcentrifuge tube.
11. Add 100 μ l of a 1:100 dilution of anti-BrdU Ab. Incubate at RT for 30 min. Add 1.2 ml PBT. Pellet at 3200 rpm for 2 min in a microcentrifuge (Eppendorf Centrifuge 5415 C). Decant supernatant. Note: Speed and time are IMPORTANT -- at this point, the pellet is very loose and it may be necessary to re-pellet.
12. Add 150 μ l of a 1:20 dilution of FITC-conjugate Ab. Incubate at RT for 30 min. Add 1.2 ml PBT. Pellet at 3200 rpm for 2 min in a microcentrifuge.
13. Resuspend pellet in 0.5 ml PI stock solution. Transfer sample to a 0.5 ml snap-top microcentrifuge tube. Cover samples with foil.
14. Leave samples at RT for 1 hr before taking them to the Flow Lab.

Reference:

Hoy CA, Seamer LC, Schimke RT. Thermal denaturation of DNA for immunohistochemical staining of incorporated bromodeoxyuridine (BrdU): critical factors that affect the amount of fluorescence and the shape of the BrdU/DNA histogram. *Cytometry*, 10: 718-725, 1989.