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BrdU Staining Kit for Flow Cytometry

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Introduction

The eBioscience BrdU Staining Kit for Flow Cytometry contains the necessary reagents and buffers for identifying and examining proliferating cells by flow cytometric analysis. Cycling cells are incubated with 5-bromo-2'-deoxyuridine (BrdU), a synthetic analog of thymidine which incorporates into newly synthesized genomic DNA during the S-phase of mitosis. Following DNA denaturation, the cells are stained for BrdU incorporation along with any other cell surface and/or intracellular targets of interest.

Protocol: *In vitro* labeling and staining of BrdU

Materials Provided

- BrdU (32.5 mM, 10 mg/mL): 5 x 1 mL vials; store at less than or equal to -80°C. Avoid multiple freeze-thaws. Vial should be opened under sterile conditions.
- DNase I (1 mg/mL; 0.3 mg/vial): 10 x 0.3 mL vials; store at less than or equal to -80°C. Each vial can be used to treat 10 samples. Avoid multiple freeze-thaws.
- Anti-BrdU Antibody (clone BU20A) fluorochrome conjugated: 1 x 100 test vial; store at 2-8°C.
- BrdU Staining Buffer Concentrate (4X): 1 x 30 mL bottle; store at 2-8°C. This buffer contains formaldehyde. Please handle appropriately.
- Fixation/Permeabilization Diluent: 1 x 100 mL bottle; store at 2-8°C.

Other Materials Needed

- Sterile 1X PBS
- Deionized water
- Flow Cytometry Staining Buffer (eBioscience Cat. No. 00-4222)
- 12 x 75 mm round bottom test tubes
- Optional:
  - Primary antibodies (directly conjugated)
  - Fixable Viability Dye (FVD) eFluor® 450 (eBioscience Cat. No. 65-0863), eFluor® 660 (eBioscience Cat. No. 65-0864), eFluor® 780 (eBioscience Cat. No. 65-0865), and eFluor® 506 (eBioscience Cat. No. 65-0866)

BrdU Staining Buffer Working Solution Preparation

- Prepare fresh 1X BrdU Staining Buffer working solution by diluting BrdU Staining Buffer Concentrate (1 part) with Fixation/Permeabilization Diluent (3 parts). Mix by gentle inversion, not vortexing. You will need 1 mL of the 1X BrdU Staining Buffer working solution for each sample. Use caution and handle appropriately as the buffer contains formaldehyde.

Note: The antibodies used for surface staining can be added after BrdU and FVD labeling (but before fixation). Alternatively if the antibody(s) is known to recognize a formaldehyde-fixed epitope, it can be added concurrently with the BrdU antibody.

Experimental Procedure

1. *In vitro* labeling of 10^5 to 10^8 dividing cells with 10 µM BrdU for 45 min at 37°C.

a) Under sterile conditions, thaw BrdU on ice and dilute to a working concentration of 1 mM with sterile 1X PBS.

b) Add 10 µM BrdU to each sample. (For example, add 10 µL of 1 mM BrdU directly to every milliliter of tissue culture medium.)

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c) Incubate your cells long enough to allow incorporation of BrdU. The timing will be dependent on your culture conditions (e.g., stimulants used) and the proliferation kinetics of your cells. Therefore, the incubation time must be determined empirically. After the incubation, harvest the cells.
d) Wash with 2 mL of Flow Cytometry Staining Buffer (or azide-free PBS if using a Fixable Viability Dye) and centrifuge at 300-400 x *g* for 5 minutes at room temperature. Decant the supernatant.

2. Optional: Stain with Fixable Viability Dye (FVD) to label dead cells before fixation.

Note: Allow the vial of Fixable Viability Dye to equilibrate to room temperature before opening. The dye must be used with azide-free and serum/protein-free PBS. For consistent staining of cells, do not stain in less than 0.5 mL. (Proceed to Step #3 if an FVD will not be used.)

a) Wash cells one additional time in azide-free PBS.
b) Resuspend cells at 1-10x10^6/mL in azide-free PBS.
c) Add 1 µL of Fixable Viability Dye per 1 mL of cells and vortex immediately.
d) Incubate for 30 minutes at 2-8°C in the dark.
e) Wash cells 1-2 times with Flow Cytometry Staining Buffer.
f) Resuspend cells at 1-10x10^6/mL in Flow Cytometry Staining Buffer.

3. Optional: Stain cell surface antigen(s).

Note: For additional information, please refer to the eBioscience website Best Protocols (Staining Cell Surface Antigens for Flow Cytometric Analysis). (Proceed to Step #4 if cell surface antigens will not be examined or if the antibody(s) is known to recognize a formaldehyde-fixed epitope.)

a) Aliquot 50 µL of cell suspension to each tube or well. The cell number should be determined empirically but can range from 10^5 to 10^8 cells/test per tube.
b) Add the recommended amount (refer to the specific Technical Data Sheet) of each fluorochrome-conjugated primary antibody(s) in an appropriate volume of Flow Cytometry Staining Buffer such that the final staining volume is 100 µL. (For example, add to 50 µL of an antibody mix to 50 µL of cells.) Mix gently.
c) Incubate for at least 30 minutes in the dark at 2-8°C.
d) Wash the cells by adding 2 mL of Flow Cytometry Staining Buffer. Pellet the cells by centrifugation at 300-400 x *g* at 4°C for 5 minutes. Repeat for a total of two washes, discarding the supernatant between washes.

4. Fix and stain with Anti-BrdU.

a) If cells were not stained in Steps 2 or 3, aliquot 100 µL of cell suspension to each tube. The cell number should be determined empirically but can range from 10^5 to 10^8 cells/tube.
b) Gently resuspend the cells (from Step 2f or 3d) by pulse-vortexing once in the residual volume after decanting (or 100 µL from Step 4a). This resuspension step is critical before addition of the freshly prepared 1X BrdU Staining Buffer working solution.
c) Add 1 mL of freshly prepared 1X BrdU Staining Buffer working solution and mix gently. Incubate for 15 minutes at room temperature in the dark. Incubations can go longer (up to 14 hours) but should be tested empirically on your cell type.
d) Centrifuge samples at 300-400 x *g* at room temperature for 5 minutes, then discard the supernatant.
e) Resuspend the cell pellet in 1 mL of 1X Flow Cytometry Staining Buffer per tube.
f) Centrifuge samples at 300-400 x *g* at room temperature for 5 minutes, then discard the supernatant.
g) During the incubation, thaw the DNase I solution on ice. To make a working solution, add 300 µL of the DNase I solution to 700 µL of Flow Cytometry Staining Buffer and mix gently. Add 100 µL of the working solution to each sample. Incubate for 1 hour at 37°C in the dark.

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h) Add 1 mL of 1X Flow Cytometry Staining Buffer to each tube.
i) Centrifuge samples at 300-400 x *g* at room temperature for 5 minutes, then discard the supernatant.
j) Resuspend the cells in 100 µL of 1X Flow Cytometry Staining Buffer per tube. Add 5 µL of Anti-BrdU fluorochrome conjugated antibody per sample. Mix and incubate for 20-30 minutes at room temperature in the dark.
k) [Optional] Antibodies against additional cell surface and/or intracellular antigens can be added here if not already added at Step #3. The primary antibodies used for cell surface staining at this step must be capable of recognizing a fixed epitope. If an antibody only recognizes a native epitope or this information is unknown, we recommend surface staining at Step #3.
l) Add 1 mL of 1X Flow Cytometry Staining Buffer to each tube.
m) Centrifuge samples at 300-400 x *g* at room temperature for 5 minutes, then discard the supernatant.
n) Resuspend stained cells in an appropriate volume of Flow Cytometry Staining Buffer and acquire data on a flow cytometer.

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