EdU Flow Cytometry Assay Kits (HF488)

Protocol

Preparing reagents

1.1 Allow vials to warm to room temperature before opening.

1.2 To prepare a 10 mM solution of EdU, add 2 mL of DMSO (Component C) or aqueous solution (PBS) to Component A and mix well. After use, store any remaining stock solution at ≤–20°C. When stored as directed, the stock solution is stable for up to 1 year.

1.3 To prepare a working solution of HyperFluor™ 488 Azide, add 260 µL of DMSO (Component C) to Component B and mix well.

1.4 To make a 10X stock solution of the EdU buffer additive (Component E), add 2 mL of deionized water buffer to the vial and mix until the EdU buffer additive is fully dissolved. After use, store any remaining stock solution at ≤–20˚C. When stored as directed, the stock solution is stable for up to 1 year.

Materials required but not provided

A. 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS), pH 7.1–pH 7.4

B. Buffered saline solution, such as PBS, D-PBS, or TBS

C. Deionized water

D. 12 × 75-mm tubes, or other flow cytometry tubes

E. 4% paraformaldehyde in PBS

F. saponin-based permeabilization and wash reagent (for example: PBS(ph7.4) + 1%FBS (or BSA) + 0.1% NaN3 + 0.1% saponin)

Labeling cells with EdU

In initial experiments, we recommend testing a range of EdU concentrations to determine the optimal concentration for your cell type and experimental conditions. If currently using a BrdU based assay for cell proliferation, a similar concentration to BrdU is a good starting concentration for EdU. If using whole blood as the sample, we recommend heparin as the anticoagulant for collection.

2.1. Suspend the cells in an appropriate tissue culture medium to obtain optimal conditions for cell growth.

Disturbing the cells by temperature changes or washing prior to incubation with EdU slows the growth of the cells during incorporation.

2.2. Add EdU to the culture medium at the desired final concentration and mix well. We recommend a starting concentration of 10 μM for 1–2 hours. For longer incubations, use lower concentrations. For shorter incubations, higher concentrations may be required. For a negative staining control, include cells from the same population that have not been treated with EdU.

2.3. Incubate under conditions optimal for cell type for the desired length of time. Altering the amount of time the cells are exposed to EdU or subjecting the cells to pulse labeling with EdU allows the evaluation of various DNA synthesis and proliferation parameters. Effective time intervals for pulse labeling and the length of each pulse depend on the cell growth rate.

2.4. Harvest cells and proceed immediately to step 3.1 if performing antibody surface labeling; otherwise continue to step 4.1.

Staining cell-surface antigens with antibodies (optional)

3.1 Wash cells once with 3 mL of 1% BSA in PBS, pellet cells by centrifugation, and remove supernatant.

3.2 Dislodge the pellet and resuspend cells at 1 × 107 cells/mL in 1% BSA in PBS.

3.3 Add 100 μL of cell suspension or whole blood sample to flow tubes.

3.4 Add surface antibodies and mix well.

Note: Do not use PE, PE-tandem, or Qdot® antibody conjugates before performing the click reaction; wait until step 6.1 for labeling with these fluorophores.

3.5 Incubate for the recommended time and temperature, protected from light.

3.6 Proceed to step 4.1 for cell fixation.

Fixation and permeabilization

Transfer the coverslips into a 6-well plate for convenient processing, such that each well contains a single coverslip.

4.1 Wash the cells once with 3 mL of 1% BSA in PBS, pellet the cells, and remove the supernatant.

4.2 Dislodge the pellet, add 100 μL of 4% paraformaldehyde in PBS, and mix well.

4.3 Incubate the cells for 15 minutes at room temperature, protected from light.

4.4 Wash the cells with 3 mL of 1% BSA in PBS, pellet the cells, and remove the supernatant. Repeat the wash step if red blood cells or hemoglobin are present in the sample. Remove all residual red blood cell debris and hemoglobin before proceeding.

4.5 Dislodge the cell pellet and resuspend the cells in 100 μL of 1×saponin-based permeabilization

and wash reagent, and mix well. Incubate the cells for 15 minutes or proceed directly to step 5.1 for click reaction.

Note: This permeabilization maintains the morphological light scatter characteristics of leukocytes while lysing red blood cells.

Click reaction

5.1 Prepare 1X EdU buffer additive by diluting the 10X stock solution (prepared in step 1.3) 1:10 in deionized water.

5.2 Prepare the Click reaction cocktail. Use this within 15 minutes after preparation.

Reaction components	Number of coverslips			
	1	2	5	10
PBS, D-PBS, or TBS	438 µL	875 µL	2.19 mL	4.38 mL
CuSO4 (Component D)	10 µL	20 µL	50 µL	100 µL
HyperFluor™ 488 azide (Component B)	2.5 µL	5 µL	12.5 µL	25 µL
EdU buffer additive (prepared in step 5.1)	50 µL	100 µL	250 µL	500 µL
Total reaction volume	500 µL	1 mL	2.5 mL	5 mL

5.3 Add 0.5 mL of reaction cocktail to each tube and mix well. (You can adjust the volume of cocktail as your preference according to previous experiments.)

5.4 Incubate the reaction mixture for 30 minutes at room temperature, protected from light.

5.5 Wash the cells once with 3 mL of saponin-based permeabilization and wash reagent, pellet the cells, and remove the supernatant. Dislodge the cell pellet and resuspend the cells in 100 µL of saponin-based permeabilization and wash reagent, if proceeding with intracellular antibody labeling in step 6.1.

Otherwise, add 500 µL of saponin-based permeabilization and wash reagent and proceed with step 7.1 for staining the cells for DNA content, or with step 8.1 for analyzing the cells on a flow cytometer.

Staining intracellular or surface antigens (optional)

6.1 Add antibodies against intracellular antigens or against surface antigens that use RPE, PE-tandem, or Qdot® antibody conjugates. Mix well.

6.2 Incubate the tubes for the time and temperature required for antibody staining, protected from light.

6.3 Wash each tube with 3 mL of saponin-based permeabilization and wash reagent, pellet the cells, and remove the supernatant. Dislodge the cell pellet and resuspend the cells in 500 µL of saponin-based permeabilization and wash reagent in PBS, and proceed with step 7.1 for staining the cells for DNA content, or with step 8.1 for analyzing the cells on a flow cytometer.

Staining cells for DNA content (optional)

7.1 If necessary, add Ribonuclease A to each tube and mix (Table 4).

7.2 Add the appropriate DNA stain such as Propidium iodide (PI) to each tube, mix well, and incubate as recommended for each DNA stain.

Analysis by flow cytometry

If measuring total DNA content on a traditional flow cytometer using hydrodynamic focusing, use a low flow rate during acquisition. If using the Attune® Acoustic Focusing Cytometer, all collection rates may be used without loss of signal integrity if the event rate is kept below 10,000 events per second. However, for each sample within an experiment, the same collection rate and cell concentration should be used. The fluorescent signal generated by DNA content stains is best detected with linear amplification. The fluorescent signal generated by EdU labeling is best detected with logarithmic amplification.

8.1 Analyze the cells using a flow cytometer.

Cy3 azide (Excitation maximum: 555 nm, Emission maximum: 570 nm)

Cy5 azide (Excitation maximum: 646 nm, Emission maximum: 662 nm)

HyperFluor™ 488 azide (Excitation maximum: 496 nm, Emission maximum: 516 nm)

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