EdU Plus Cell Proliferation Protocols Flow Cytometry

Introduction

Many commercial EdU-based kits used for detecting proliferating cells provide great results, but are often expensive and provide fixed amounts of reagents, which limits optimized or off-protocol use of these kits. “Home-made” kits are a viable alternative to commercially available kits, in particular when all of the components are widely available from a number of suppliers. The amounts of reagents and the click reaction conditions are very similar between many commercial kits, and are in line with large number of published procedures for EdU-based detection of proliferating cells. Using the provided protocols, a researcher will be able to assemble an EdU-based cell proliferation assay that would require very little, if any, fine tuning.

EdU Plus kits with improved biocompatibility and detection limits were first commercialized by ThermoFisher Scientific and sold under Click-iT® Plus EdU assays label. The introduction of a copper-chelating moiety at the azide reporter molecule allows for a dramatic increase of the effective Cu(I) concentration at the reaction site, enhancing the weakest link in the reaction rate acceleration, greatly increasing the sensitivity biocompatibility of EdU-based assays for analyzing DNA replication in proliferating cells.

Materials Required

- EdU Reagent
- AFDye Picolyl Azide
- Copper (II) Sulfate pentahydrate
- THPTA
- Sodium ascorbate
- Fixative (4% paraformaldehyde in PBS)
- Permeabilization and wash reagent (0.1% Saponin, 0.002% NaN_3, 1% BSA in PBS)
- 1% BSA in PBS (pH 7.4)

Material Preparation

EdU Stock Solution	Prepare 20 mM solution of EdU in DMSO or water, for example to make 1 mL of 20 mM stock solution dissolve 5 mg in 1 mL of DMSO or Water
AFDye Picolyl Azide Stock Solution	Prepare 295 µM solution in DMSO or water. Example: to make 140 µL dissolved the entire AFDye Picolyl Azide Kit Pack in 140 µL of DMSO or water
Copper Catalyst (50 mM $CuSO_4$, 125 mM THPTA) solution	Weight out 624 mg of Copper (II) Sulfate Pentahydrate and 2.7 g of THPTA, mix, add 50 mL of water, vortex to dissolve completely
Reducing Agent	Dissolve 20 mg of sodium ascorbate in 1 mL of deionized water. Vortex until completely dissolved. Sodium ascorbate solution is susceptible to oxidation. We recommend always using freshly prepared solution of sodium ascorbate.
Permeabilization and wash reagent	(0.1% Saponin, 0.002% NaN_3 in PBS): Add 1 g of saponin and 0.02 g of dry sodium azide to 1 L of PBS

1. Cell labeling with EdU

This protocol is based on a large number of publications of EdU-based procedures for analyzing DNA replication in proliferating cells used with different types of cells. An optimized EdU concentration is around 10 µM, but may need slight adjustment depending on the given cell type. Growth medium, cell density, cell type variations, and other factors may influence labeling. Investigators are encouraged to determine the optimal concentration of the EdU reagent as well as labeling time individually for each cell type on a small-scale first. Metabolic labeling is a critical step for successful cell proliferation and should be carefully assessed for each cell line of interest.

1. Suspend the cells in an appropriate 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. Prepare 20 mM solution of EdU in DMSO or water
3. Add desired amount of EdU to cells in culture medium to achieve optimal working EdU concentration (10 µM, if not optimizes). We have found a final concentration of 10 µM EdU to be sufficient for labeling of most cell lines.
4. During addition of EdU to cells in culture, avoid disturbing the cells in ways that may disrupt the normal cell cycling patterns.
5. Incubate the cells for the desired length of time under conditions optimal for the cell type. Different cell types may require different incubation periods for optimal labeling with EdU. As a starting point we recommend 10 µM EdU for 1-2 hour. 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.
6. Proceed immediately to **Cell fixation and permeabilization or cell-surface antigen staining with antibodies (optional)**.

(Optional) Cell-surface antigen staining with antibodies

7. After EdU labeling wash cells once with 3 mL of 1% BSA in PBS, pellet cells by centrifugation, and remove supernatant.
8. Dislodge the pellet and resuspend cells at 1×10^7 cells/mL in 1% BSA in PBS.
9. Add surface antibodies, mix well and incubate for the recommended time and temperature protected from light.
10. Proceed to cell fixation step.

2. Cell fixation and permeabilization

The following protocol is provided for fixation step using saponin-based permeabilization and wash reagent. This reagent can be used with whole blood or cell suspensions containing red blood cells, as well as with cell suspensions containing more than one cell type. This permeabilization and wash reagent maintains the morphological light scatter characteristics of leukocytes while lysing red blood cells. Protocols using other fixation/permeabilization reagents, such as methanol followed by a 0.5% Triton® X-100 permeabilization step also can be used.

1. Wash the cells once with 3 mL of 1% BSA in PBS, pellet the cells, and remove the supernatant.
2. Dislodge the pellet, add 100 µL of Fixative and mix well, and incubate for 15 minutes.
3. Remove the fixative and wash the cells in each with 1 mL of 1% BSA in PBS. If red blood cells or hemoglobin are present in the sample repeat the wash step. If require remove all residual cell debris.
4. Remove the wash solution and resuspend the cells in 100 µL of saponin-based permeabilization and wash reagent, and mix well. Incubate the cells for 15 minutes.
5. Proceed directly to EdU detection step or to treating cells with antibodies to intracellular antigens followed by EdU detection step.

3. EdU detection

Note: A smaller volume can be used as long as the remaining reaction components are maintained at the same ratios.

1. Prepare the required amount of the reaction cocktail according to Table 1. **Add the ingredients in the order listed in the table**. Use the reaction cocktail within 15 minutes of preparation.

Table 1

Reaction component	Number of coverslips					
	1	2	5	10	15	50
PBS Buffer	438 µL	875 µL	2.19 mL	4.38 mL	6.57 mL	21.9 mL
Copper Catalyst (Material preparation)	10 µL	20 µL	50 µL	100 µL	150 µL	500 mL
Picolyl Azide Solution (Material preparation)	2.5 µL	5 µL	12.5 µL	25 µL	37.5 µL	125 µL
Reducing Agent (Material preparation)	50 µL	100 µL	250 µL	500 µL	750 µL	2.5 mL
Total Volume	500 µL	1 mL	2.5 mL	5 mL	7.5 mL	25 mL

2. Remove the permeabilization buffer (step 2.4).
3. Add 0.5 mL of the **Reaction Cocktail** to each tube and mix.
4. **Protect from light**, and incubate the plate for 30 minutes at room temperature.
5. Remove the reaction cocktail. Wash the cells well once with 3 mL of saponin-based permeabilization and wash reagent. Pellet the cells and remove the wash solution.
6. If intracellular antibody labeling required dislodge the cell pellet and resuspend the cells in 100 µL of saponin-based permeabilization and wash reagent.
7. If no intracellular antibody labeling required, add 500 µL of saponin-based permeabilization and wash reagent.
   At this point the samples are ready for **DNA staining**. If no **DNA staining** is desired, proceed to analyzing the cells on a flow cytometer.

4. (Optional) intracellular or surface antigens staining

1. Add antibodies against intracellular antigens or against surface antigens that use RPE, PR-tandem or Quantum Dot antibody conjugates. Mix well.
2. Protect from light and incubate the tubes for the time and temperature required for antibody staining.
3. If **EdU Detection** has not yet performed, wash the cells once with 3 mL of 1% BSA in PBS, pellet the cells, and remove the supernatant. Dislodge the cell pellet and add 100 µL of saponin-based permeabilization and wash reagent, mix well and proceed to **EdU Detection** (step 3.1)
4. If **EdU Detection** has been performed (steps 3.1–3.7), 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, and proceed to DNA staining, or to the cell analysis on a flow cytometer.

5. DNA staining

1. Add the appropriate DNA stain to each tube and mix well.

2. Protect from light and incubate as recommended by a manufacture
3. Remove the DNA stain and wash each tube with 3 mL of saponin-based permeabilization and wash reagent each well.

Flow Cytometry Analysis

Use a low flow rate during acquisition, if a traditional flow cytometer with a hydrodynamic focusing is used to measure the total DNA content. The same collection rate and cell concentration should be used for each sample within an experiment. Detect the fluorescent signal generated by DNA content stains with linear amplification. The fluorescent signal generated by EdU labeling is best detected with logarithmic amplification.
The Excitation and emission maxima of the available dyes are listed in **table 2**.

Table 2

	Excitation (nm)	Emission (nm)
PB Picolyl Azide	**403**	**453**
AFDye 350 Azide	**350**	**440**
AFDye 488 Azide	**495**	**519**
AFDye 555 Azide	**550**	**570**
AFDye 594 Azide	**590**	**617**
AFDye 647 Azide	**648**	**671**