EdU Imaging Kits (Cy3)

Introduction

Measuring cell proliferation and cell cycle are a fundamental method to assess cell health, determine genotoxicity, and evaluate drug's pharmacodynamic effect. The common method is measuring DNA synthesis directly. In previous experiments, there are several approaches such as the incorporation of radioactive nucleosides (3H-thymidine) or BrdU. Here, we introduce one new method, click chemistry-CuAAC (Copper-Catalyzed Azide-Alkyne Cycloaddition), and the use of this reaction in direct measurement of S-phase DNA synthesis in cell cycle.

A nucleoside analog of thymidine, EdU (5-ethynyl-2'-deoxyuridine), can be incorporated into DNA strand during DNA synthesis. The alkynyl group of EdU is a biologically inert group that will undergo an extremely selective reaction with dye's azido via a CuAAC reaction to afford an 1,2,3-triazole product. EdU and Cy3 azide possess biologically unique moieties to label DNA of proliferating cells, producing low backgrounds and high detection sensitivities. This CuAAC reaction affords superior regioselectivity and quantitative transformation under extremely mild conditions.

EdU Imaging Kits (Cy3) specifically labels the DNA of proliferating cells after Cy3 azide is ligated to EdU, then the proliferating cells can be detected by a microscope or flow cytometry.

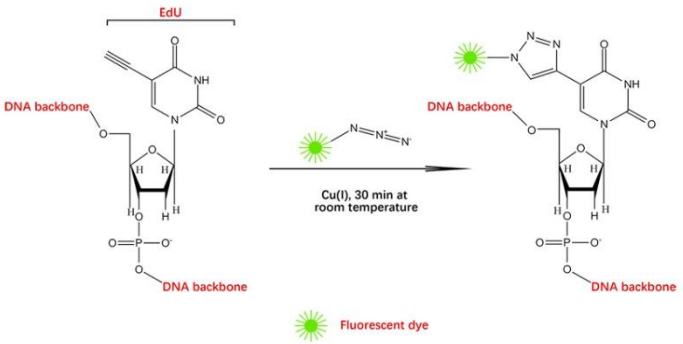


Figure 1. Click reaction(CuAAC) between EdU and azide-modified dye

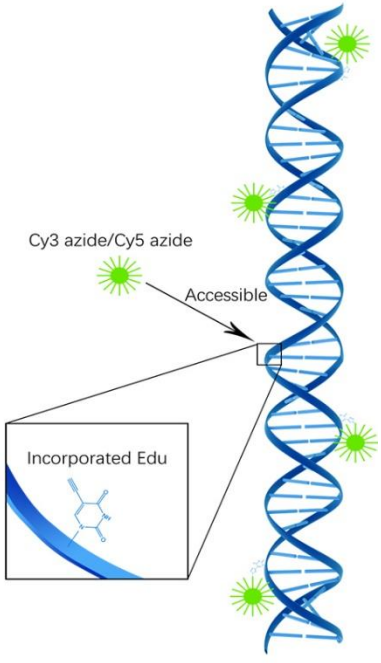


Figure 2. Detection of the incorporated EdU with Cy3/Cy5 azide

Components and Storage

Components	50-500 Tests	Storage
EdU (Component A)	5 mg	-20°C
Cy3 Azide (Component B)	1 vial	-20°C away from light
DMSO (Component C)	4 mL	Room temperature
10X EdU Reaction Buffer (Component D)	4 mL	-20°C
$CuSO_4$ (100 mM Aqueous Solution) (Component E)	1 vial	-20°C
EdU Buffer Additive (Component F)	400mg	-20°C
Hoechst 33342 (10 mg/mL in Water) (Component G)	35 μL	-20°C away from light
Shipping: Blue ice	Shelf life: 1 year	

Protocol

1. Preparing reagents

1) Allow vials to warm to room temperature before opening.

2) Prepare 10 mM EdU stock solution: Add 2 mL of DMSO (Component C) or aqueous solution (buffer, PBS, or saline) 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.

3) Prepare Cy3 Azide working solution: Add 25 μL of DMSO (Component C) to Component B and mix well.

4) Prepare 1X EdU reaction buffer: Dilute 10X EdU Reaction Buffer (Component D) in deionized water to make the 1X EdU reaction buffer. After use, store any remaining 1X solution at 2–6ºC. When stored as directed, this 1X solution is stable for up to 6 months.

5) Prepare 10X stock solution of the EdU buffer additive: Add 2 mL of deionized water to the vial of EdU Buffer Additive (Component F) and mix until the EdU buffer additive is fully dissolved. After use, store any remaining stock solution at -20ºC. When stored as directed, the stock solution is stable for up to 1 year. If the solution develops a brown color, it has degraded and should be discarded.

2. Materials required but not provided

A. Phosphate-buffered saline (PBS, pH 7.2–7.6)

B. Fixative (for example, 4% formaldehyde in PBS)

C. Permeabilization reagent (for example, 0.3% Triton X-100 in PBS)

D. 3% BSA in PBS, pH 7.4

E. Deionized water

3. Protocol for fluorescence microscopy

1. 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 you are currently using a BrdU-based assay for cell proliferation, a similar concentration to BrdU is a good starting concentration for EdU.

1) Plate the cells at the desired density in a 6-well plate, then allow them to recover overnight before additional treatment.

2) Prepare a 2X working solution of EdU in a complete medium from the 10 mM stock solution. You can take a final concentration of 10 μM as a start point. (For example, for a final concentration of 10 μM, prepare a 2X working solution of 20 μM).

3) Prewarm the 2X EdU solution, then add an equal volume of the 2X EdU solution to the volume of media containing cells to be treated to obtain a 1X EdU solution. (For example, for a final concentration of 10 μM, replace half of the media with fresh media containing 20 μM of EdU). We do not recommend replacing all of the media, because this could affect the rate of cell proliferation.

4) Incubate the cells for the desired length of time under conditions optimal for the cell type. We recommend using 2 h as a starting time. The choice of time points and the length of time depends on the cell growth rate.

2. Fixation and permeabilization

1) After incubation, remove the media and add 1 mL of 4% formaldehyde in PBS to each well. Incubate for 15 min at room temperature.

2) Remove the fixative and wash the cells in each well twice with 1 mL of 0.3% BSA in PBS, 3-5 min per time.

3) Remove the wash solution. Add 1 mL of 0.3% Triton X-100 in PBS to each well, then incubate at room temperature for 10-15 min.

4) Wash the cells in each well twice with 1 mL of 0.3% BSA in PBS.

3. Click reaction

1) Prepare 1X EdU buffer additive by diluting the 10X stock solution 1:10 in deionized water. Prepare this solution fresh and use the solution on the same day.

2) Prepare the Click reaction cocktail. Use this within 15 min after preparation.

Components	6-well plate			
	1	2	4	5
1X EdU Reaction Buffer	430 μL	860 μL	1.8 mL	2.2 mL

CuSO4 (Component E)	20 μL	40 μL	80 μL	100 μL
Cy3 azide	0.5 μL	1 μL	2 μL	2.5 μL
1X EdU Buffer Additive	50 μL	100 μL	200 μL	250 μL
Total volume	500 μL	1 mL	2 mL	2.5 mL

3) Add 500 μL of click reaction cocktail to each well. (You can adjust the volume of the cocktail to your preference according to previous experiments.) Rock the plate briefly to ensure that the reaction cocktail is distributed evenly.

4) Incubate the plate for 30 min at room temperature, protected from light.

5) Remove the reaction cocktail, then wash each well once with 1 mL of 0.3% BSA in PBS. Remove the wash solution.

6) Optional: Perform antibody labeling of the samples at this time. It is important to keep the samples protected from light during incubations.

4. **DNA staining**

1) Wash each well with 1 mL of PBS. Remove the wash solution.

2) Dilute the Hoechst 33342 (Component G) solution 1:2000 in PBS to obtain a 1X Hoechst 33342 solution (the final concentration is 5 μg/mL).

3) Add 1 mL of 1X Hoechst 33342 solution per well. Incubate for 15 min at room temperature, protected from light. Remove the Hoechst 33342 solution.

4) Wash each well twice with 1 mL of PBS. Remove the wash solution.

5. **Imaging**

1) Proceed to detection in a fluorescence with filters for Cy3 (Ex/Em: 555/570 nm) and Hoechst 33342 (Ex/Em: 350/461 nm).

4. Protocol for flow cytometry

1. **Labeling cells with EdU**

1) Harvest 0.1-1 x 10^6 cells in culture medium in sterile flow test tubes or centrifuge tubes.

2) Prepare a 2X working solution of EdU in a complete medium from the 10 mM stock solution. You can take a final concentration of 10 μM as a start point. (For example, for a final concentration of 10 μM, prepare a 2X working solution of 20 μM).

3) Prewarm the 2X EdU solution, then add an equal volume of the 2X EdU solution to the volume of cells to be treated to obtain a 1X EdU solution.

4) Incubate the cells for the desired length of time under conditions optimal for the cell type. We

recommend using 2 h as a starting time. The choice of time points and the length of time depends on the cell growth rate.

5) Centrifuge to pellet cells and remove supernatant.

2. **Fixation and permeabilization**

1) Wash cells once in PBS by centrifugation at 500 x g for 5 min and discard the supernatant.

2) Add 1 mL of 4% formaldehyde in PBS to each tube to suspend cells. Incubate for 15 min at room temperature.

3) Remove the fixative by centrifugation and wash the cells twice with 1 mL of 0.3% BSA in PBS.

4) Centrifuge to remove the wash solution. Add 1 mL of 0.3% Triton X-100 in PBS to each tube and incubate at room temperature for 10-15 min. Centrifuge to discard the supernatant.

5) Wash the cells twice in 1 mL of 0.3% BSA in PBS by centrifugation at 500 x g for 5 min.

3. **Click reaction**

1) Prepare 1X EdU buffer additive by diluting the 10X stock solution 1:10 in deionized water. Prepare this solution fresh and use the solution on the same day.

2) Prepare the Click reaction cocktail. Use this within 15 min after preparation.

Components	Samples number			
	1	2	4	5
1X EdU Reaction Buffer	430 μL	860 μL	1.8 mL	2.2 mL
CuSO4 (Component E)	20 μL	40 μL	80 μL	100 μL
Cy3 Azide (Component B)	1 μL	2 μL	4 μL	5 μL
1X EdU Buffer Additive	50 μL	100 μL	200 μL	250 μL
Total volume	501 μL	1 mL	2 mL	2.5 mL

3) Remove the wash buffer, add 500 μL of click reaction cocktail per tube to resuspend cells. (You can adjust the volume of the cocktail to your preference according to previous experiments.)

4) Incubate for 30 min at room temperature, protected from light. Then remove the reaction cocktail by centrifugation at 500 x g for 5 min.

5) Wash cells once with 1 mL of 0.3% BSA in PBS by centrifugation at 500 x g for 5 min and discard the supernatant. Add 500 μL of PBS per tube to resuspend cells.

6) Optional: Perform antibody labeling of the samples at this time. It is important to keep the samples protected from light during incubations.

4. **Detection**

1) Proceed to analyze on a flow cytometer (Ex/Em: 555/570 nm).

Note

1. If more EdU is required for animal experiments, it can be purchased by our catalog number (B8337 5-EdU).
2. For your safety and health, please wear lab coats and gloves during the experiment.
3. For research use only. Not to be used in clinical diagnostic or clinical trials.

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