# EdU Cell Proliferation Assay Protocol for Fluorescent Microscopy

## Introduction

Many commercial EdU-based kits used for detecting proliferating cells provide great results, but are often quite expensive and provide fixed amounts of reagents, which limits optimized or off-protocol use of these kits. Self-assembled 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.

## Materials Required

- EdU Reagent
- AFDye Azide
- Copper (II) Sulfate pentahydrate
- Sodium ascorbate
- Permeabilization reagent (for example, 0.5% solution of Triton®X-100 in PBS)
- Fixative (3.7% formaldehyde in PBS)
- 3% BSA in PBS (pH 7.4)
- Coverslips/microscope slides
- Mounting media
- Hoechst 33342 (optional)

## 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 Azide Stock Solution** | Prepare 2 mM solution in DMSO or water. Example: to make 70 µL dissolved the entire AFDye Azide Kit Pack in 70 µL of DMSO or water |
| **25 mM copper (II) sulfate solution** | Weight out 312 mg of Copper (II) Sulfate Pentahydrate, add 50 mL of water, vortex to dissolve completely |
| **Reaction Buffer** | 50 mM Tris, 150 mM NaCl, pH 7.5. Dissolve 3.02 g of Tris, 4.4 g of NaCl in 500 mL of water, adjust pH to 7.5, sterile filter |
| **Hoechst 33342** | 10 mg/mL stock solution. Dissolve 1 mg of Hoechst 33342 in 100 µL of deionized water |
| **Reducing Agent** | Dissolve 20 mg of sodium ascorbate in 1.8 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. |
| **Wash buffer** | (0.5 mM EDTA, 2 mM $NaN_3$ in PBS): Add 1 mL of 0.5 M EDTA and 0.13 g of dry sodium azide to 1 L of PBS. Sterile filter for long term storage |

## 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. Plate the cells on coverslips at the desired density and allow them to recover overnight before additional treatment.
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 optimized).
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 hour.
6. Proceed immediately to **Cell fixation and permeabilization**.

## 2. Cell fixation and permeabilization

The following protocol is provided for the fixation step using 3.7% formaldehyde in PBS followed by a 0.5% Triton® X-100 permeabilization step. Protocols using other fixation/permeabilization reagents, such as methanol and saponin, can also be used.

1. Transfer each coverslip into a single well. For convenient processing, use 6-well plates.
2. After metabolic labeling, remove the media and add 1 mL of 3.7% formaldehyde in PBS to each well containing the coverslips. Incubate for 15 minutes at room temperature.
3. Remove the fixative and wash the cells in each well twice with 1 mL of 3% BSA in PBS.
4. Remove the wash solution. Add 1 mL of 0.5% Triton® X-100 in PBS to each well, then incubate at room temperature for 20 minutes.

## 3. EdU detection

Note: 500 µL of the reaction cocktail is used per coverslip. 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**

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| Reaction component | Number of coverslips | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 4 | 5 | 10 | 25 | 50 |
| **Reaction Buffer** (Material preparation) | 430 µL | 860 µL | 1.7 mL | 2.2 mL | 4.3 mL | 10.7 mL | 21.4 mL |
| **Copper (II) Sulfate** (Material preparation) | 20 µL | 40 µL | 80 µL | 100 µL | 200 µL | 500 µL | 1 mL |

| Reaction component | Number of coverslips | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 4 | 5 | 10 | 25 | 50 |
| AFDye Azide Solution (Material preparation) | 1.2 µL | 2.5 µL | 5 µL | 6 µL | 12.5 µL | 31 µL | 62 µL |
| Reducing Agent (Material preparation) | 50 µL | 100 µL | 200 µL | 250 µL | 500 µL | 1.25 mL | 2.5 mL |
| Total Volume | 500 µL | 1 mL | 2.0 mL | 2.5 mL | 5.0 mL | 12.5 mL | 25 mL |

2. Remove the permeabilization buffer (step 2.4).
   Wash the cells in each well twice with 1 mL of 3% BSA in PBS.
   Remove the wash solution.
3. Add 0.5 mL of the **Reaction Cocktail** to each well containing a coverslip.
   Rock the plate briefly to insure that the reaction cocktail is distributed evenly over the coverslip.
4. **Protect from light**, and incubate the plate for 30 minutes at room temperature.
5. Remove the reaction cocktail.
   Wash each well once with 1 mL of 3% BSA in PBS.
   Remove the wash solution.
6. Wash each well once with 1 mL of **Wash Buffer**.
   Remove the wash solution.
7. Wash each well once with 1 mL of PBS.
   Remove the wash solution.

   At this point the samples are ready for **DNA staining**. If no **DNA staining** is desired, proceed to **Imaging**.

   If antibody labeling of the samples is desired, proceed to labeling according to manufacturer's recommendations. **Keep the samples protected from light during incubation**.

## 4. DNA staining

1. Wash each well with 1 mL of PBS. Remove the wash solution.
2. Prepare 1x **Hoechst 33342 solution** by diluting stock solution of **Hoechst 33342** 1:2000. The final concentration of 1x **Hoechst 33342 solution** is 5 µg/mL.
   Final concentrations of 1x **Hoechst 33342** may range from 2 µg/mL to 10 µg/mL.
3. Add 1 mL of **1x Hoechst 33342 solution** per well. **Protected from light**. Incubate for 30 minutes at room temperature.
4. Remove the Hoechst 33342 solution.
5. Wash each well twice with 1 mL of PBS.
6. Remove the wash solution.

## Imaging

Labeled cells are compatible with all methods for slide preparation. See **Table 2** for approximate fluorescence excitation/emission maxima for Fluorescent Azides and Hoechst 33342 dye bound to DNA.

**Table 2**

| | Excitation (nm) | Emission (nm) |
|---|---|---|

| | Excitation (nm) | Emission (nm) |
|---|---|---|
| PB Azide | 405 | 450 |
| AFDye 350 Azide | 350 | 440 |
| AFDye 405 Azide | 402 | 461 |
| AFDye 488 Azide | 495 | 519 |
| AFDye 555 Azide | 550 | 570 |
| AFDye 594 Azide | 590 | 617 |
| AFDye 650 Azide | 650 | 670 |
| Hoechst 33342 bound to DNA | 350 | 461 |