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# Click-&-Go™ Plus EdU Flow Cytometry Kit, 50 Assays

## Product No. 1373, 1375, 1377, 1379, 1381, 1383

## Introduction

Click-&-Go™ Plus EdU is a next step in improving the biocompatibility and sensitivity of traditional EdU-based proliferation assays. The copper concentrations typically used in traditional click chemistry reactions can affect fluorophores such as green fluorescent protein, mCherry, and R-phycoerythrin and results in loss of fluorescence signal. The advantage of Click-&-Go Plus EdU assay is that it employs the newest generation of copper-chelating azides, Azide Plus probes, that can form a strong copper-azide complex, and thus dramatically rising the copper concentration at the reaction site without the need to maintain overall copper concertation at high level. As a result much lower copper concentration and copper protectants can be used without losing efficiency of copper-catalyzed detection reaction. This makes Click-&-Go Plus EdU assay compatible with cell cycle dyes, R-PE, R-PE tandems, and fluorescent proteins such as GFP, RFP, and mCherry (Table 1). The Click-&-Go Plus EdU assay can also be multiplexed with antibodies against surface and intracellular markers.

Table 1: Compatibility of Click-&-Go Plus assay with detection reagents.

| Molecule | Compatibility |
|---|---|
| R-phycoerythrin (R-PE) and R-PE based tandems (e.g., Alexa Fluor™ 610-RPE) | Compatible, except Alexa Fluor™ 594 with R-PE-Cy®7 tandems |
| PerCP, allophycocyanin (APC) and APC-based tandems (e.g. Alexa Fluor™ 680-APC) | Compatible |
| Fluorescent proteins (e.g. GFP, mCherry) | Compatible |
| Nanocrystals | Not compatible, should be used **after** EdU detection. |

## Kit contents

| Component | Concentration | Amount |
|---|---|---|
| EdU (Component A) | - | 10 mg |
| Fluorescent Azide Plus (Component B) | - | 1 vial |
| Fixative (Component C) | 4% PBS solution | 4 mL |
| Copper Catalyst (Component D) | 50X solution | 0.55 mL |
| Reducing Agent (Component E) | - | 400 mg |
| Saponin-based permeabilization and wash reagent (Component F) | 10X solution | 50 mL |

## Materials required but not provided

- 1% BSA in PBS (pH 7.4)
- Flow cytometry tubes
- PBS buffer, pH 7.4
- Deionized water

## Additional information

- Fixative (Component C) contains paraformaldehyde, which is harmful. Use with appropriate precautions.
- Saponin-based permeabilization and wash reagent (Component F) contains sodium azide, which yields highly toxic hydrazoic acid under acidic conditions. Dilute azide compounds in running water before discarding to avoid accumulation of potentially explosive deposits in plumbing.

## Material preparation

**EdU (Component A)**

Add 2 mL of DMSO to make 20 mM stock solution. This solution is stable up to 1 year if stored at -20C protected from light.

**Fluorescent Azide Plus (Component B)**

Add 140 μL of DMSO or water. This solution is stable up to 1 year if stored at -20C protected from light.

**1X saponin-based permeabilization and wash reagent (Component F)**

To prepare 500 mL of 1X saponin-based permeabilization and wash reagent, add 50 mL of **Component F** to 450 mL of 1% BSA in PBS. Smaller amounts can be prepared by diluting a volume of **Component F** 1:10 with 1% BSA in PBS. After use, store any remaining solutions at 2–8°C. When stored as directed, the 1X solution is stable for 6 months and the 10X solution is stable for 12 months after receipt.

| | |
|---|---|
| **Reducing Agent (Component E)** | Prepare **1 x solution** of **Reducing Agent (Component E)** that is enough for one day. Weigh our 20 mg of **Reducing Agent (Component E)** into 2 mL vial, add 1 mL of deionized water. Vortex until completely dissolved.<br><br>Note- reducing agent is susceptible to oxidation and turns brown when oxidized. If solution appears brown do not use. |

## Cell labeling with EdU

This protocol was successfully tested with HEK 293T, HeLa, and NIH/3T3 cells with an optimized EdU concentration of 10 µM, it can be easily adapted for any adherent cell type. Growth medium, cell density, cell type variations, and other factors may influence labeling. Investigators may wish to determine the optimal concentration of EdU reagent as well as labeling time individually for each cell type on a small-scale first. Metabolic labeling is critical step for successful cell proliferation and should be carefully assessed for each cell line of interest.

1.1 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.

1.2 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.

1.3 During addition of EdU to cells in culture, avoid disturbing the cells in ways that may disrupt the normal cell cycling patterns.

1.4 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.

1.5 Proceed immediately to **Cell fixation and permeabilization** or **cell-surface antigen staining with antibodies (optional).**

## Optional cell-surface antigen staining with antibodies

1.6 After EdU labeling wash cells once with 3 mL of 1% BSA in PBS, pellet cells by centrifugation, and remove supernatant.

1.7 Dislodge the pellet and resuspend cells at $1 \times 10^7$ cells/mL in 1% BSA in PBS.

1.8 Add surface antibodies, mix well and incubate for the recommended time and temperature protected from light.

1.9 Proceed to cell fixation step.

## 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 and 3.7% formaldehyde in PBS followed by a 0.5% Triton®X-100 permeabilization step also can be used.

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

2.2 Dislodge the pellet, add 100[1] µL of Fixative (Component C) and mix well, and incubate for 15 minutes.

2.3 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.

2.4 Remove the wash solution and resuspend the cells in 100 µL of 1X saponin-based permeabilization and wash reagent, and mix well. Incubate the cells for 15 minutes.

2.5 Proceed directly to EdU detection step or to treating cells with antibodies to intracellular antigens followed by EdU detection step.

## EdU detection

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

3.1 Weight our 20 mg of **Reducing Agent (Component E)** into 2 mL vial, add 1 mL of deionized water, vortex until completely dissolved. This solution should be used freshly prepared on the same day.

3.2 Prepare required amount of the reaction cocktail according to Table 2. **Add the ingredients in the order listed in the table**. Use the reaction cocktail within 15 minutes of preparation.

**Table 2.**

| Reaction component | Number of reactions | | | | | |
|---|---|---|---|---|---|---|
| | **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 (Component D) | 10 µL | 20 µL | 50 µL | 100 µL | 150 µL | 500 µL |
| Fluorescent Azide Plus solution (Component B) | 2.5 µL | 5 µL | 12.5 µL | 25 µL | 37.5 µL | 125 µL |
| 1 x Reducing Agent (step 3.1) | 50 µl | 100 µL | 250 µL | 500 µL | 750 µL | 2.5 mL |

[1]

| | | | | | | |
|---|---|---|---|---|---|---|
| Total Volume | 500 µL | 1 mL | 2.5 mL | 5 mL | 7.5 mL | 25 mL |

3.3 Remove the permeabilization buffer (step 2.5).

3.4 Add 0.5 mL of the **Reaction Cocktail** to each tube. Rock the plate briefly to insure that the reaction cocktail is distributed evenly.

3.5 **Protect from light**, and incubate the plate for 30 minutes at room temperature.

3.6 Remove the reaction cocktail.
Wash the cells well once with 3 mL of 1X saponin-based permeabilization and wash reagent.
Pallet the cells and remove the wash solution.

3.7 If intracellular antibody labeling required dislodge the cell pellet and resuspend the cells in 100 µL of 1X saponin-based permeabilization and wash reagent.

3.8 If no intracellular antibody labeling required add 500 µL of 1X 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.

## Optional intracellular or surface antigens staining

4.1 Add antibodies against intracellular antigens or against surface antigens that use RPE, PR-tandem or Quantum Dot antibody conjugates. Mix well.

4.2 Protect from light and incubate the tubes for the time and temperature required for antibody staining.

4.3 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 1x saponin-based permeabilization and wash reagent, mix well and proceed to **EdU Detection** (step 3.1)

4.4 If **EdU Detection** has been performed (steps 3.1–3.8), wash each tube with 3 mL of 1X 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 1X saponin-based permeabilization and wash reagent, and proceed to DNA staining, or to the cell analysis on a flow cytometer.

## DNA staining

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

5.2 Protect from light and incubate as recommended by a manufacture.

5.3 Remove the DNA stain and wash each tube with 3 mL of 1X 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 3.

**Table 3.**

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