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Click-&-Go™ Plus EdU Cell Proliferation Kit for Imaging

Product No. 1347, 1348, 1349, 1350, 1351, 1352, 1353

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. Sufficient amounts of reagents are provided for imaging 50 (18x18) coverslips using 500 µL of reaction buffer per test.

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)	-	5 mg
Fluorescent Azide Plus (Component B)	-	1 vial
Reaction Buffer (Component C)	10X solution	4 mL
Copper Catalyst (Component D)	25X solution	1.2 mL
Reducing Agent (Component E)	-	400 mg
Hoechst 33342 (Component F)	10 mg/mL	50 µL
Wash Buffer (Component G)	n/a	50 mL

Materials required but not provided

- Fixative (for example, 3.7% Formaldehyde in PBS)
- Permeabilization reagent (for example, 0.5% solution of Triton® X-100 in PBS)
- 3% BSA in PBS (pH 7.4)
- Coverslips/microscope slides, mounting media
- PBS buffer, pH 7.4
- Deionized water

Additional information

Hoechst 33342 (Component F) is a known mutagen. Use the dye with appropriate precautions.

Material preparation

EdU (Component A)

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

Fluorescent Azide Plus (Component B)

Add 70 µL of DMSO or water. This solution is stable up to 1 year if stored at -20C and protected from light.

Reaction Buffer (Component C)

To prepare the required amount of **1x reaction buffer** (see Table 1), dilute the appropriate volume from the **Reaction Buffer** (**Component C)** bottle 1:10 with deionized water. To convert the entire amount of **10x Reaction buffer** into 1x working solution, transfer the entire bottle of **10x Reaction Buffer** (4 mL) into 36 mL of deionized water. Store undiluted 10X reaction buffer at 2–8°C. The 10X solution is stable for 1 year.

Reducing Agent (Component E)	Prepare enough **1 x solution** of **Reducing Agent (Component E)** for one day. Weigh out 20 mg of **Reducing Agent (Component E)** into 2 mL vial, and add 1.8 mL of deionized water. Vortex until completely dissolved. Note- The reducing agent is susceptible to oxidation and turns brown when oxidized. We recommend always using freshly prepared reducing agent solution.

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, but it can be easily adapted for any adherent cell type. Growth medium, cell density, cell type variations, and other factors may influence labeling. However, 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 a critical step for successful cell proliferation and should be carefully assessed for each cell line of interest.

1.1 Plate the cells on coverslips at the desired density and allow them to recover overnight before additional treatment.

1.2 Prepare 20 mM solution of EdU in DMSO or water (use 1 mL of solvent per 5 mg of EdU)

1.3 Add desired amount of EdU to cells in culture medium to achieve optimal working EdU concentration (10 µM, if not optimized). We have found a final concentration of 10 µM EdU to be sufficient for labeling of most cell lines.

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

1.5 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.

1.6 Proceed immediately to **Cell fixation and permeabilization**.

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.

2.1 Transfer each coverslip into a single well. For convenient processing, use 6-well plates.

2.2 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.

2.3 Remove the fixative and wash the cells in each well twice with 1 mL of 3% BSA in PBS.

2.4 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.

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.

3.1 Weigh out 20 mg of **Reducing Agent (Component E)** into 2 mL vial, add 1.8 mL of deionized water, vortex until completely dissolved. This solution should be freshly prepared and used on the same day.

3.2 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**	**4**	**5**	**10**	**25**	**50**
1x 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 (Component D)	20 µL	40 µL	80 µL	100 µL	200 µL	500 µL	1 mL
Azide Plus Solution (Material preparation)	1.2 µL	2.5 µL	5 µL	6 µL	12.5 µL	31 µL	62 µL
1x Reducing Agent (step 2.1)	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

3.3 Remove the permeabilization buffer (step 1.4).
Wash the cells in each well twice with 1 mL of 3% BSA in PBS.
Remove the wash solution.

3.4 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.

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

3.6 Remove the reaction cocktail.
Wash each well once with 1 mL of 3% BSA in PBS.
Remove the wash solution.

3.7 Wash each well once with 1 mL of **Wash Buffer (Component G)**.
Remove the wash solution.

3.8 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.**

DNA staining

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

4.2 Prepare 1x **Hoechst 33342 solution** by diluting stock solution of **Hoechst 33342 (Component F)** 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.

4.3 Add 1 mL of **1x Hoechst 33342 solution** per well. **Protected from light.** Incubate for 30 minutes at room temperature.

4.4 Remove the Hoechst 33342 solution.

4.5 Wash each well twice with 1 mL of PBS.

4.6 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)
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 647 Azide	650	670
Hoechst 33342 bound to DNA	350	461