molecular probes
by life technologies

Click-iT® Plus EdU Imaging Kits

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Table 1 Contents and storage

Material	C10637	C10638	C10639	C10640	Concentration	Storage*
EdU (Component A)	5 mg	5 mg	5 mg	5 mg	NA	• 2–8°C • Desiccate • Protect from light • DO NOT FREEZE
Alexa Fluor® picolyl azide (Component B)	1 vial of Alexa Fluor® 488 picolyl azide (70 µL)	1 vial of Alexa Fluor® 555 picolyl azide (70 µL)	1 vial of Alexa Fluor® 594 picolyl azide (70 µL)	1 vial of Alexa Fluor® 647 picolyl azide (70 µL)	DMSO solution	
Dimethylsulfoxide (DMSO) (Component C)	4 mL	4 mL	4 mL	4 mL	NA	
Click-iT® EdU reaction buffer (Component D)	4 mL	4 mL	4 mL	4 mL	10X solution containing Tris-buffered saline	
Copper protectant (Component E)	1 vial, 0.5 mL	1 vial, 0.5 mL	1 vial, 0.5 mL	1 vial, 0.5 mL	NA	
Click-iT® EdU buffer additive (Component F)	400 mg	400 mg	400 mg	400 mg	NA	
Hoechst® 33342 (Component G)	35 µL	35 µL	35 µL	35 µL	10 mg/mL in water	

*These storage conditions are appropriate when storing the entire kit upon receipt. For optimal storage conditions for each component, see vial labels. When stored as directed the product is stable for 1 year.
NA = Not applicable.

Number of assays: Sufficient material is supplied for 50 coverslips based on the protocol below.

Approximate fluorescence excitation and emission maxima, in nm: Alexa Fluor® 488 picolyl azide**:** 495/519; Alexa Fluor® 555 picolyl azide**:** 555/565; Alexa Fluor® 594 picolyl azide: 590/615; Alexa Fluor® 647 picolyl azide: 650/670; Hoechst® 33342: 350/461, bound to DNA.

Introduction

Measuring a cell's ability to proliferate is a fundamental method for assessing cell health, determining genotoxicity, and evaluating anti-cancer drugs. The most accurate method is by directly measuring DNA synthesis. Initially, this was performed by incorporation of radioactive nucleosides (for example, ^{3}H-thymidine). This method was replaced by antibody-based detection of the nucleoside analog bromo-deoxyuridine (BrdU). The Click-iT® Plus EdU Assay is a novel alternative to the BrdU assay. EdU (5-ethynyl-2´-deoxyuridine) provided in the kit is a nucleoside analog of thymidine and is incorporated into DNA during active DNA synthesis[1]. Detection is based on a click reaction,[2-5] a copper catalyzed covalent reaction between a picolyl azide and an alkyne. In this application, the EdU contains the alkyne and the Alexa Fluor® dye contains the

picolyl azide. The advantages of the Click-iT® Plus EdU labeling are readily evident while performing the assay. The small size of the dye picolyl azide allows for efficient detection of the incorporated EdU using mild conditions. Standard aldehyde-based fixation and detergent permeabilization are sufficient for the Click-iT® Plus detection reagent to gain access to the DNA. This is in contrast to BrdU assays that require DNA denaturation (typically using HCl, heat, or digestion with DNase) to expose the BrdU so that it may be detected with an anti-BrdU antibody (Figure 1, below).

The denaturation step in the BrdU protocol can disrupt dsDNA integrity (which can affect nuclear counterstaining), and can also destroy cell morphology and antigen recognition sites. In contrast, the EdU assay kit is easy to use and fully compatible with DNA staining, including dyes for cell cycle analysis. This EdU assay kit can also be multiplexed with fluorescent proteins (for example, GFP, RFP, and mCherry; see Table 2 on page 3 for details). Unlike the BrdU assay, which relies upon antibodies which can exhibit nonspecific binding, the Click-iT® Plus EdU assay utilizes bioorthogonal (biologically unique) moieties, producing low backgrounds and high detection sensitivities.

The kit contains all of the components needed to label and detect the incorporated EdU, as well as perform cell cycle analysis on samples from adherent cells (Figure 2, page 3). For cell cycle analysis, the kit is supplied with blue fluorescent Hoechst® 33342 dye. The kits include sufficient reagents for labeling 50, 18 × 18 coverslips using 500 µL reaction buffer per test.

Figure 1 Detection of the incorporated EdU with the Alexa Fluor® picolyl azide versus incorporated BrdU with an anti-BrdU antibody. The small size of the Alexa Fluor® picolyl azide eliminates the need to denature the DNA for the EdU detection reagent to gain access to the nucleotide.

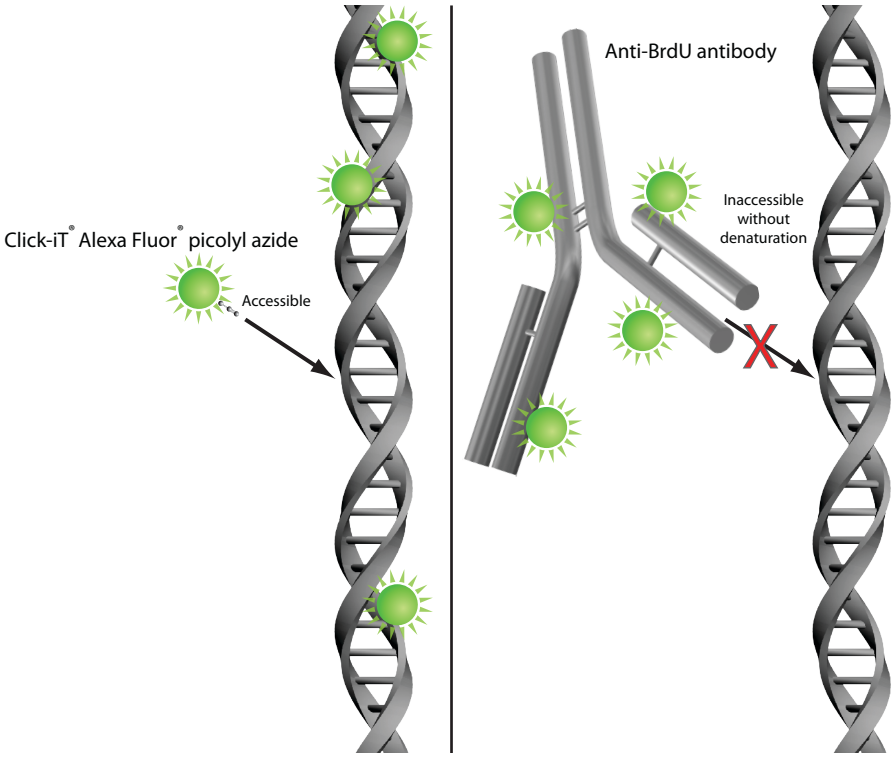
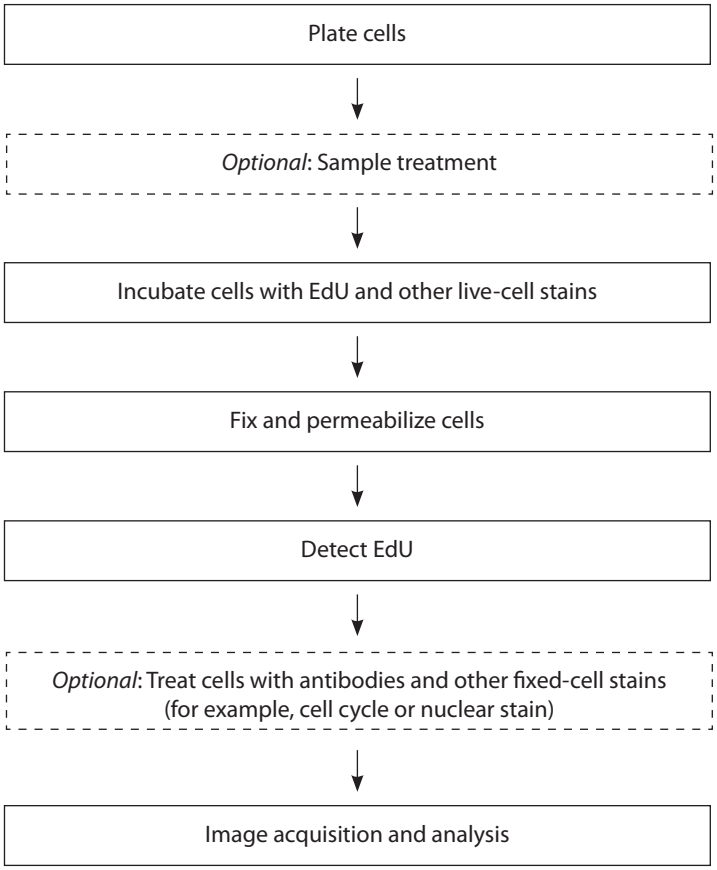


Table 2 Click-iT® Plus detection reagent compatibility.

Molecule	Compatibility*
Qdot® nanocrystals	Use Qdot® nanocrystals **after** the Click-iT® Plus detection reaction.
Fluorescent proteins such as Green Fluorescent Protein (GFP)	**Completely compatible** with the Click-iT® Plus detection reaction.
Organic dyes such as Alexa Fluor® dyes, fluorescein (FITC)	**Completely compatible** with the Click-iT® Plus detection reaction.
Phalloidin	Phalloidin staining is **not compatible** with the Click-iT® Plus detection reaction. Use antibodies against other proteins, such as anti-α-tubulin, for visualization of the cytoskeleton.
Horseradish peroxidase (HRP)	Use HRP **after** the Click-iT® Plus detection reaction.
R-phycoerythrin (R-PE) and R-PE-tandems such as Alexa Fluor® 680-R-PE	**Completely compatible** with the Click-iT® Plus detection reaction.
Allophycocyanin (APC) and APC-tandems	**Completely compatible** with the Click-iT® Plus detection reaction.

*Compatibility indicates whether the fluorescent molecule itself or the detection method involves components that are unstable in the presence of copper catalyst and copper protectant used for the Click-iT® Plus detection reaction.

Figure 2 Workflow diagram for the Click-iT® Plus EdU Imaging Assay.



Before You Begin

Materials required but not provided

- Phosphate buffered saline (PBS, pH 7.2–7.6)
- Fixative (i.e., 3.7% Formaldehyde in PBS)
- Permeabilization reagent (i.e., 0.5% Triton® X-100 in PBS)
- 3% Bovine serum albumin (BSA) in PBS (3% BSA in PBS), pH 7.4
- Deionized water
- 18 × 18-mm coverslips
- Optional: 6-well microplate

Cautions

- Hoechst® 33342 (Component G) is a known mutagen. Use the dye with appropriate precautions.
- DMSO (in Components B and C), provided as a solvent in this kit, is known to facilitate the entry of organic molecules into tissues. Handle reagents containing DMSO using equipment and practices appropriate for the hazards posed by such materials. Dispose of the reagents in compliance with all pertaining local regulations.

Prepare stock solutions

1.1 Allow the vials to warm to room temperature before opening.

1.2 Prepare a 10 mM solution of EdU (Component A) by adding 2 mL DMSO (Component C) or an aqueous solution (i.e., buffer, saline) to EdU (Component A). 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.

1.3 Prepare a working solution of 1X Click-iT® EdU reaction buffer (Component D): Transfer the solution (4 mL) in the Component D bottle to 36 mL of deionized water.

To make smaller amounts of 1X Click-iT® EdU reaction buffer, dilute volumes from the Component D bottle 1:10 with deionized water. After use, store any remaining 1X solution at 2–8°C. When stored as directed, this 1X solution is stable for 6 months.

1.4 To make a 10X stock solution of the Click-iT® EdU buffer additive (Component F): Add 2 mL deionized water to the vial, then mix until fully dissolved. After use, store any remaining stock solution at ≤–20°C.

When stored as directed, this stock solution is stable for up to 1 year. If the solution develops a brown color, it has degraded and should be discarded.

Label Cells with EdU

The following protocol was developed with A549, HeLa, and NIH/3T3 cells with an optimized EdU concentration of 10 µM, but it can be adapted for any adherent cell type. Growth medium, cell density, cell type variations, and other factors may influence labeling. In initial experiments, we recommend testing a range of EdU concentrations to determine the optimal concentration for your cell type and experimental conditions. Although sufficient material is included with the kit for standard dose response, additional EdU (Cat. nos. A10044, E10187) is available. If currently using a BrdU based assay for cell proliferation, a similar concentration to BrdU is a good starting concentration for EdU.

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

2.2 Prepare a 2X working solution of EdU (Component A) in complete medium from the 10 mM stock solution. A suggested starting concentration is 10 µM.

2.3 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 (e.g., for a final concentration of 10 µM, replace half of the media with fresh media containing 20 µM EdU). We do not recommend replacing all of the media, because this could affect the rate of cell proliferation.

2.4 Incubate the cells for the desired length of time under conditions optimal for the cell type. The time of EdU exposure to the cells allows for direct measurement of cells synthesizing DNA. The choice of time points and the length of time depends on the cell growth rate. Pulse labeling of cells by brief exposures to EdU permits studies of cell-cycle kinetics.

2.5 Proceed immediately to **Fix and permeabilize cells** (steps 3.1–3.3, below) followed by **Detect EdU** (steps 4.1–4.7, page 6).

Fix and permeabilize cells

Note: This protocol is optimized with a fixation step using 3.7% formaldehyde in PBS followed by a 0.5% Triton® X-100 permeabilization step, but it is also amenable to other fixation/permeabilization reagents such as methanol and saponin.

Transfer the coverslips into a 6-well plate for convenient processing such that each well contains a single coverslip.

3.1 After incubation, 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.

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

3.3 Remove the wash solution. Add 1 mL of 0.5% Triton® X-100 in PBS to each well and incubate for 20 minutes at room temperature.

Detect EdU

Note: This protocol uses 500 µL of Click-iT® Plus reaction cocktail per coverslip. A smaller volume can be used as long as the remaining reaction components are maintained at the same ratios.

4.1 Prepare 1X Click-iT® EdU buffer additive (see Table 3, below) by diluting the 10X solution (prepared in step 1.4) 1:10 in deionized water. Prepare this solution **fresh** and use the solution on the same day.

4.2 Prepare Click-iT® Plus reaction cocktail according to Table 3 below. It is important to add the ingredients in the order listed in the table; otherwise, the reaction will not proceed optimally.

Note: Use the Click-iT® Plus reaction cocktail within 15 minutes of preparation.

Table 3 Click-iT® Plus reaction cocktails.

Reaction components*	Number of coverslips						
	1	2	4	5	10	25	50
1X Click-iT® reaction buffer (prepared in step 1.3)	440 µL	880 µL	1.84 mL	2.25 mL	4.4 mL	10.9 mL	21.9 mL
Copper protectant (Component E)	10 µL	20 µL	40 µL	50 µL	100 µL	250 µL	500 µL
Alexa Fluor® picolyl azide (Component B)	1.2 µL	2.5 µL	5 µL	6 µL	12.5 µL	31 µL	62 µL
Reaction buffer additive (prepared in step 4.1)	50 µL	100 µL	200 µL	250 µL	500 µL	1.25 mL	2.5 mL
Total volume	500 µL	1 mL	2 mL	2.5 mL	5 mL	12.5 mL	25 mL

*__Note__: Add the ingredients in the order listed in the table.

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

4.4 Add 0.5 mL of Click-iT® Plus reaction cocktail to each well containing a coverslip. Rock the plate briefly to insure that the reaction cocktail is distributed evenly over the coverslip.

4.5 Incubate the plate for 30 minutes at room temperature, **protected from light**.

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

For nuclear staining, proceed to **Stain DNA** below. If no additional staining is desired, proceed to **Image and Analyze** on page 7.

4.7 *Optional*: Perform antibody labeling of the samples at this time, following the recommendations from the manufacturer of the primary and secondary antibody. It is important to keep the samples **protected from light** during incubations.

Stain DNA

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

5.2 Dilute the Hoechst® 33342 (Component G) solution 1:2,000 in PBS to obtain a 1X Hoechst® 33342 solution (final concentration is 5 µg/mL).

Note: A range between 2–10 µg/mL of Hoechst® 33342 has been shown to work.

5.3 Add 1 mL of 1X Hoechst® 33342 solution per well. Incubate for 30 minutes at room temperature, **protected from light**. Remove the Hoechst® 33342 solution.

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

Image and analyze

Click-iT® Plus EdU cells are compatible with all methods of slide preparation including wet mount or prepared mounting media. See Table 4 below for the approximate fluorescence excitation/emission maxima for Alexa Fluor® dyes and Hoechst® 33342 dye bound to DNA.

Table 4 Approximate fluorescence excitation/emission maxima.

Fluorophore	Excitation (nm)	Emission (nm)
Alexa Fluor® 488 picolyl azide	495	519
Alexa Fluor® 555 picolyl azide	555	565
Alexa Fluor® 594 picolyl azide	590	615
Alexa Fluor® 647 picolyl azide	650	670
Hoechst® 33342, bound to DNA	350	461

References

1. Proc Natl Acad Sci USA 105, 2415 (2008); **2.** ChemBioChem 4, 1147 (2003); **3.** J Am Chem Soc 125, 3192 (2003); **4.** Angew Chem Int Ed Engl 41, 2596 (2002); **5.** Angew Chem Int Ed Engl 40, 2004 (2001).

Product List Current prices may be obtained from our website or from our Customer Service Department.

	Cat. no.	Product Name	Unit Size
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Buy Now	E10187	EdU (5-ethynyl-2´-deoxyuridine)	500 mg
Buy Now	E10415	EdU (5-ethynyl-2´-deoxyuridine)	5 g
Buy Now	H3570	Hoechst® 33342, trihydrochloride, trihydrate *10 mg/mL solution in water*	10 mL

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