

Click-iT™ EdU Alexa Fluor® Imaging Kits

Table 1. Contents and storage information.

Material	C10083	C10084	C10085	Concentration	Storage*	Stability
EdU (Component A)	5 mg	5 mg	5 mg	NA	<ul style="list-style-type: none"> • 2–6°C • Desiccate • Protect from light • DO NOT FREEZE 	When stored as directed this kit is stable for 1 year
Alexa Fluor® azide (Component B)	1 vial (Alexa Fluor® 488)	1 vial (Alexa Fluor® 594)	1 vial (Alexa Fluor® 647)	NA		
Dimethylsulfoxide (DMSO, Component C)	4 mL	4 mL	4 mL	NA		
Click-iT™ EdU reaction buffer (Component D)	4 mL	4 mL	4 mL	10X solution containing Tris-buffered saline		
CuSO ₄ (Component E)	1 vial	1 vial	1 vial	100 mM aqueous solution		
Click-iT™ EdU buffer additive (Component F)	400 mg	400 mg	400 mg	NA		
Hoechst 33342 (Component G)	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 labels on the vials. NA = Not applicable.

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

Approximate fluorescence excitation/emission maxima: Alexa Fluor® 488 azide: 495/519 in nm; Alexa Fluor® 594 azide: 590/615 in nm; Alexa Fluor® 647 azide: 650/670 in nm; Hoechst 33342: 350/461 in nm, 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 of doing this is by directly measuring DNA synthesis. Initially this was performed by incorporation of radioactive nucleosides, i.e., ³H-thymidine. This method was replaced by antibody-based detection of the nucleoside analog bromo-deoxyuridine (BrdU). The Click-iT™ EdU Assay from Invitrogen 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. Detection is based on a click reaction,¹⁻⁴ a copper (Cu⁺) catalyzed covalent reaction between an azide and an alkyne. In this application, the EdU contains the alkyne and the Alexa Fluor® dye contains the azide. The advantages of the Click-

iT™ EdU labeling are readily evident while performing the assay. The small size of the dye 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™ detection reagent to gain access to the DNA. This is in contrast to BrdU assays that require DNA denaturation (typically using HCl or heat or digestion with DNase) to expose the BrdU so that it may be detected with an anti-BrdU antibody (Figure 1).

Sample processing for the BrdU assay can result in signal alteration of the cell cycle distribution as well as destruction of antigen recognition sites when using the HCl method. In contrast, the EdU assay kit is not only easy to use but also compatible with cell cycle dyes. This EdU assay kit can also be multiplexed with surface and intracellular marker detection using antibodies (see Table 2 for details).

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). For cell cycle analysis, the kit is supplied with blue fluorescent Hoechst 33342 dye for cell cycle staining or cell registration. The kits include sufficient reagents for labeling 50, 18 × 18 coverslips using 500 µL reaction buffer per test.

Before You Begin

Materials Required but Not Provided

- Phosphate buffered saline (PBS, Invitrogen Cat. no. 14190-144 or 14190-250)
- 3.7% Formaldehyde in PBS
- 0.5% Triton® X-100 in PBS
- 3% Bovine serum albumin (BSA) in PBS (3% BSA in PBS), pH 7.4
- Deionized water or 18 megohm purified water
- 18 × 18 coverslips
- 6-well microplate

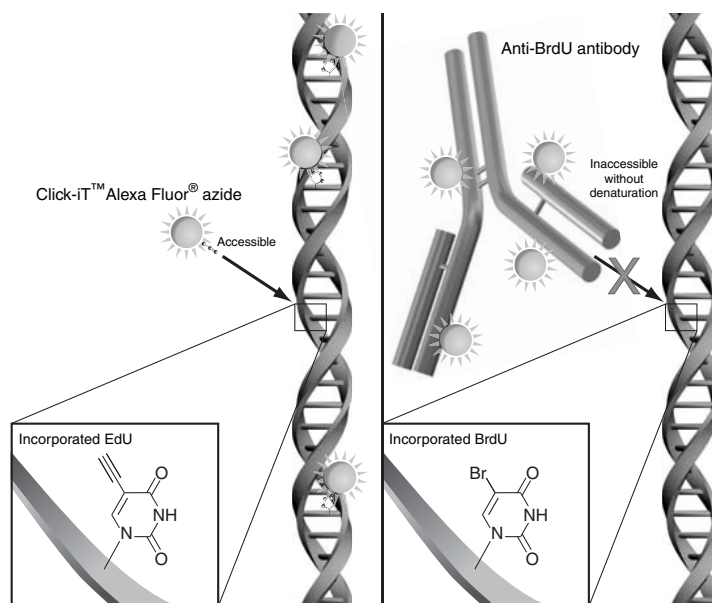


Figure 1. Detection of the incorporated EdU with the Alexa Fluor® azide versus incorporated BrdU with an anti-BrdU antibody. The small size of the Alexa Fluor® azide eliminates the need to denature the DNA for the EdU detection reagent to gain access to the nucleotide.

Table 2. Click-iT™ EdU detection reagent compatibility.

Fluorescent molecule	Compatibility*
Qdot® nanocrystals	Use Qdot® nanocrystals after the Click-iT™ detection reaction.
Fluorescent proteins (GFP)	Use organic dye-based reagents, such as TC-FIAsh™ or TC-ReAsH™ reagents, for protein expression detection or anti-GFP rabbit or chicken antibodies before the Click-iT™ detection reaction..
Organic dyes such as Alexa Fluor® dyes, fluorescein (FITC)	Completely compatible with the Click-iT™ detection reaction.
TC-FIAsh™ or TC-ReAsH™ reagents	Detect the tetracysteine (TC) tag with FIAsh™ or ReAsH™ reagents before the Click-iT™ detection reaction.

*Compatibility indicates whether the fluorescent molecule itself or the detection methods involve components that are unstable in the presence of copper catalyst used for the Click-iT™ EdU detection reaction.

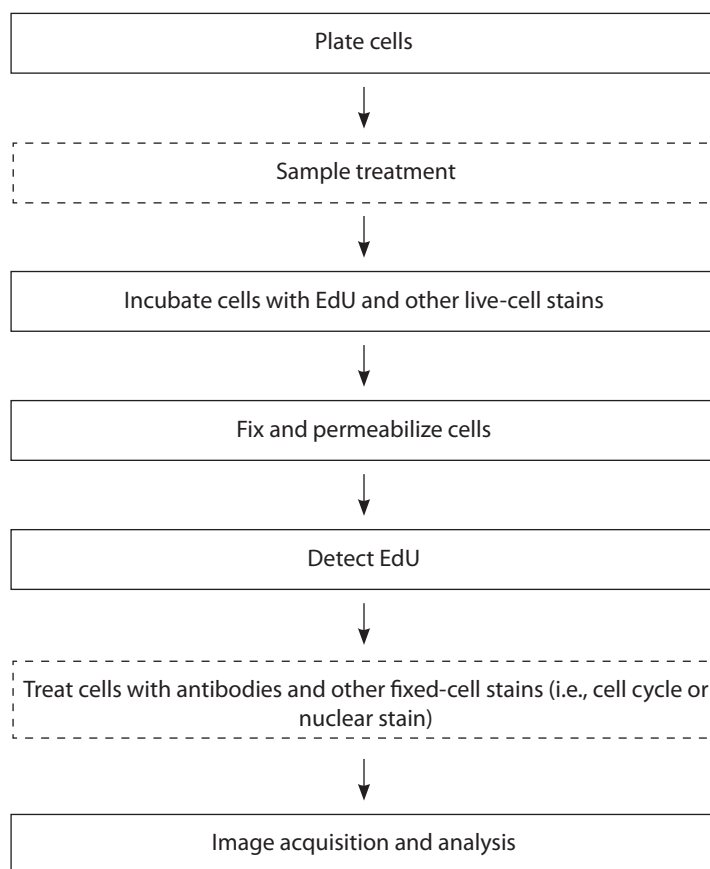


Figure 2. Workflow diagram for the Click-iT™ EdU Imaging Assay.

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

DMSO (Component 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.

Preparing the Stock Solutions

1.1 Allow 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 aqueous solution (i.e., buffer, saline) to EdU (Component A) and mix well.

After use, store any remaining stock solution at $\leq -20^{\circ}\text{C}$. When stored as directed the stock solution is stable for up to 1 year.

1.3 Prepare a working solution of the Alexa Fluor® azide, (Component B) by adding 70 μL DMSO (Component C) to Component B and mix well.

After use, store any remaining working solution at $\leq -20^{\circ}\text{C}$. When stored as directed, this working solution is stable for up to 1 year.

1.4 Prepare a working solution of 1X Click-iT™ EdU reaction buffer (Component D) by transferring all of the solution (4 mL) in the Component D bottle to 36 mL of deionized water. Rinse the Component D bottle with some of the diluted Click-iT™ EdU reaction buffer to ensure the transfer of all of the 10X concentrate.

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 $2-6^{\circ}\text{C}$. When stored as directed, this 1X solution is stable for 6 months.

1.5 To make a 10X stock solution of the Click-iT™ EdU buffer additive (Component F), add 2 mL deionized water to the vial and mix until fully dissolved. After use, store any remaining stock solution at $\leq -20^{\circ}\text{C}$. When stored as directed, this stock solution is stable for up to 1 year.

Experimental Protocols

Labeling Cells with EdU

The following protocol was developed with A549, HeLa, and NIH/3T3 cells with an optimized EdU concentration of 10 μM , but 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 (Invitrogen, Cat. no. A10044) is available. If currently using a BrdU based assay for cell proliferation, a similar concentration to BrdU will be a good starting concentration for EdU.

2.1 Plate cells on coverslips at the desired density and allow cells 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 μ M, replace half of the media with fresh media containing 20 μ M EdU). Replacement of all of the media is not recommended since this could affect the rate of cell proliferation.
- 2.4 Incubate under conditions optimal for cell type for the desired length of time. The choice of time points and length of time depends on the cell growth rate. The time of EdU exposure to the cells allows for direct measurement of cells synthesizing DNA.
- 2.5 Incubate under conditions optimal for cell type for the desired length of time. The time of EdU exposure to the cells allows for the direct measurement of cells synthesizing DNA. The choice of time points and length of time for pulsing depends on the cell growth rate. Pulse labeling of cells by brief exposures to EdU permits studies of cell-cycle kinetics.
- 2.6 Proceed immediately to **Cell Fixation and Permeabilization** (steps 3.1–3.3) followed by **EdU Detection** (steps 4.1–4.6).

Cell Fixation and Permeabilization

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 is 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 media and add 1 mL 3.7% formaldehyde in PBS to each well containing coverslips. Incubate for 15 minutes at room temperature.
- 3.2 Remove fixative and wash cells in each well twice with 1 mL 3% BSA in PBS.
- 3.3 Remove the wash solution. Add 1 mL 0.5% Triton[®] X-100 in PBS to each well and incubate for 20 minutes at room temperature.

EdU Detection

- 4.1 Prepare 1X Click-iT[™] EdU buffer additive (see Table 3) by diluting the 10X solution (prepared in step 1.5) 1:10 in deionized water. Prepare this solution **fresh** and use the solution on the same day.
- 4.2 Prepare Click-iT[™] reaction cocktail according to Table 3.

Note: Use the Click-iT[™] reaction cocktail within 15 minutes of preparation.

Table 3. Click-iT[™] reaction cocktails.

Reaction components	Number of coverslips						
	1	2	4	5	10	25	50
1X Click-iT [™] reaction buffer (prepared in step 1.4)	430 μ L	860 μ L	1.8 mL	2.2 mL	4.3 mL	10.7 mL	21.4 mL
CuSO ₄ (Component H)	20 μ L	40 μ L	80 μ L	100 μ L	200 μ L	500 μ L	1 mL
Alexa Fluor [®] azide (prepared in step 1.3)	1.2 μ L	2.5 μ L	5 μ L	6 μ L	12.5 μ L	31 μ L	62 μ L
Reaction buffer additive (prepared in step 4.1)	50 μ L	100 μ L	200 μ L	250 μ L	500 μ L	1.25 mL	2.5 mL
Total volume	500 μ L	1 mL	2 mL	2.5 mL	5 mL	12.5 mL	25 mL

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

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

Note: You may need to adjust the amount of dye azide to provide optimum signal to background.

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

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

If antibody labeling is desired, proceed to section 5. For DNA staining, proceed to section 6. If no additional staining is desired, proceed to **Imaging and Analysis**.

Antibody Detection (optional)

5.1 If required, block the plate with 1 mL/well 3% BSA in PBS for the recommended time, protected from light.

5.2 Remove the blocking solution from the wells.

5.3 Prepare primary antibodies (as recommended by the manufacturer) and add the primary antibody solution.

5.4 Incubate for the recommended time and temperature, protected from light. Remove the antibody solution.

5.5 Wash each well twice with 1 mL/well 3% BSA in PBS. Remove the wash solution.

5.6 Prepare and add the secondary antibody as recommended by the manufacturer.

5.7 Incubate for the recommended time and temperature, protected from light. Remove the antibody solution.

5.8 Wash each well twice with 1 mL/well 3% BSA in PBS. Remove the wash solution.

For DNA staining and cell cycle distribution, proceed to section 6. If no additional staining is desired, proceed to **Imaging and Analysis**.

DNA Staining

6.1 Wash each well with 1 mL/well PBS. Remove the wash solution.

6.2 Dilute Hoechst 33342 (Component G) solution 1:2000 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.

6.3 Add 1 mL/well 1X Hoechst 33342 solution. Incubate for 30 minutes at room temperature, protected from light. Remove the Hoechst 33342 solution.

6.4 Wash each well twice with 1 mL/well PBS. Remove the wash solution.

Imaging and Analysis

Click-iT™ EdU cells are compatible with all methods of slide preparation including wet mount or prepared mounting media. See Table 4 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	495	519
Alexa Fluor® 594	590	615
Alexa Fluor® 647	650	670
Hoechst 33342, bound to DNA	350	461

References

1. ChemBioChem 4, 1147 (2003); 2. J Am Chem Soc 125, 3192 (2003); 3. Angew Chem Int Ed Engl 41, 2596 (2002); 4. 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
C10083	Click-iT™ EdU Alexa Fluor® 488 Imaging Kit *50 coverslips*	1 kit
C10084	Click-iT™ EdU Alexa Fluor® 594 Imaging Kit *50 coverslips*	1 kit
C10085	Click-iT™ EdU Alexa Fluor® 647 Imaging Kit *50 coverslips*	1 kit
Related Products		
A10027	Click-iT™ EdU Alexa Fluor® 488 High-Throughput Imaging (HCS) Assay *2-plate size*	1 kit
A10028	Click-iT™ EdU Alexa Fluor® 488 High-Throughput Imaging (HCS) Assay *10-plate size*	1 kit
A10034	Click-iT™ EdU Pacific Blue Flow Cytometry Assay Kit *50 assays*	1 kit
C35002	Click-iT™ EdU Alexa Fluor® 488 Flow Cytometry Assay Kit *50 assays*	1 kit
A10202	Click-iT™ EdU Alexa Fluor® 647 Flow Cytometry Assay Kit *50 assays*	1 kit
A10209	Click-iT™ EdU Alexa Fluor® 594 High-Throughput Imaging (HCS) Assay *2-plate size*	1 kit
A10208	Click-iT™ EdU Alexa Fluor® 647 High-Throughput Imaging (HCS) Assay *2-plate size*	1 kit
C10082	Click-iT™ EdU Alexa Fluor® 594 High-Throughput Imaging (HCS) Assay *10-plate size*	1 kit
C10081	Click-iT™ EdU Alexa Fluor® 647 High-Throughput Imaging (HCS) Assay *10-plate size*	1 kit
H1399	Hoechst 33342, trihydrochloride, trihydrate	100 mg
H3570	Hoechst 33342, trihydrochloride, trihydrate *10 mg/mL solution in water*	10 mL
H21492	Hoechst 33342, trihydrochloride, trihydrate *FluoroPure™ grade*	100 mg
14190-144	Dulbecco's Phosphate Buffered Saline 1X without Calcium Chloride without Magnesium Chloride	500 mL
14190-250	Dulbecco's Phosphate Buffered Saline 1X without Calcium Chloride without Magnesium Chloride	10 × 500 mL

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