Click-iT® EdU HCS Assays

Table 1. Contents and storage information.

Material	C10350/C10351	C10352/C10353	C10354/C10355	C10356/C10357	Concentration	Storage*
EdU (Component A)	105 µL/525 µL	105 µL/525 µL	105 µL/525 µL	105 µL/525 µL	10 mM solution in DMSO	• 2–6˚C • Desiccate • Protect from light • DO NOT FREEZE
Alexa Fluor® azide (Component B)	70 µL/ 330 µL (Alexa Fluor® 488)	70 µL/ 330 µL (Alexa Fluor® 555)	70 µL/ 330 µL (Alexa Fluor® 594)	70 µL/ 330 µL (Alexa Fluor® 647)	1X DMSO solution	
Click-iT® EdU reaction buffer (Component C)	4 mL/15 mL	4 mL/15 mL	4 mL/15 mL	4 mL/15 mL	10X solution in Tris-buffered saline	
$CuSO_4$ (Component D)	1 vial	1 vial	1 vial	1 vial	100 mM aqueous solution	
Click-iT® EdU buffer additive (Component E)	400 mg	400 mg	400 mg	400 mg	NA	
Click-iT® reaction rinse buffer (Component F)	25 mL/125 mL	25 mL/125 mL	25 mL/125 mL	25 mL/125 mL	1X	
HCS NuclearMask™ Blue stain (Component G)	25 µL/ 125 µL	25 µL/ 125 µL	25 µL/ 125 µL	25 µL/ 125 µL	1,000X	

*When stored as directed, this kit is stable for 1 year from the date of receipt. These storage conditions are appropriate when storing the entire kit. For optimal storage conditions for each component, see labels on the individual vials. NA = Not applicable.

Number of assays: Sufficient material is supplied for 2 × 96-well plates (Cat. nos. C10350, C10352, C10354, C10356) or 10 × 96-well plates (Cat. nos. C10351, C10353, C10355, C10357), based on the protocol below.

Approximate fluorescence excitation/emission maxima: Alexa Fluor® 488 azide: 495/519 in nm; Alexa Fluor® 555 azide: 555/565 in nm; Alexa Fluor® 594 azide: 590/615 in nm; Alexa Fluor® 647 azide: 650/670 in nm; HCS NuclearMask™ Blue stain: 350/461 in nm, bound to DNA.

Introduction

The Click-iT® EdU HCS Assay is a superior alternative to traditional methods for detecting and quantitating newly synthesized DNA. 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 an azide and an alkyne. In this application, the EdU contains the alkyne and the Alexa Fluor® dye contains the azide. The small size of the dye azide allows for efficient detection of the incorporated EdU using mild conditions 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).

The HCl denaturation step in the BrdU detection protocol not only disrupts dsDNA integrity which can affect nuclear counterstaining, but also potentially destroys cell morphology and antigen recognition sites.

In contrast, the EdU assay is fully compatible with DNA staining, including dyes for cell cycle analysis, and can also be multiplexed with surface and intracellular marker detection using antibodies (Figure 2, Table 2). Furthermore, the streamlined Click-iT® EdU detection protocol reduces the total number of steps and decreases the time required to perform a cell proliferation assay. Unlike the BrdU assay, which relies upon antibodies that can exhibit nonspecific binding, the Click-iT® EdU assay utilizes bioorthogonal or 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 in adherent cells (Figure 3). For cell registration or DNA profiling, the kit is supplied with blue-fluorescent HCS NuclearMask™ Blue stain. The kits include sufficient reagents for performing 2 × 96-well or 10 × 96-well plates, using 100 µL of reaction buffer per assay. For custom and bulk packagings of these products, inquire at www.invitrogen.com.

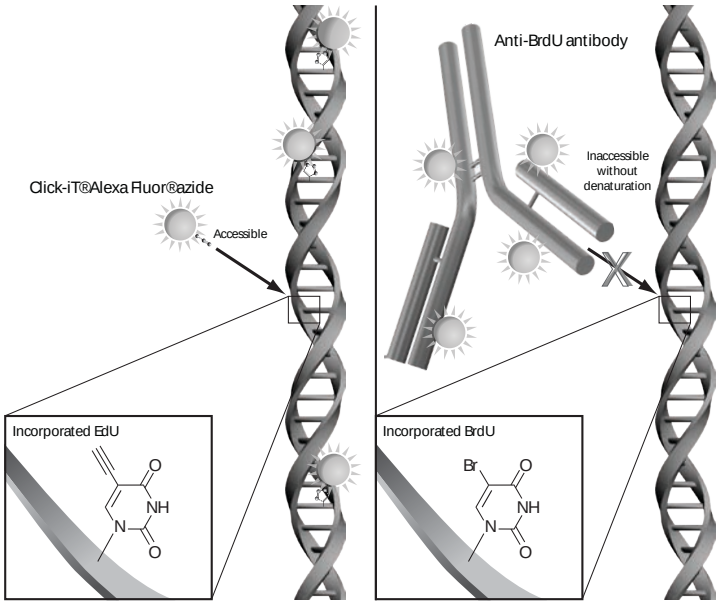

Figure 1. Detection of the incorporated EdU with the Alexa Fluor® azide versus incorporated BrdU with an anti-BrdU antibody. The small size of the Alexa Fluor® azide eliminates the need to denature the DNA for the EdU detection reagent to gain access to the nucleotide.

Table 2. Click-iT® detection reagent compatibility.

Molecule	Compatibility*
Qdot® nanocrystals	Use Qdot® nanocrystals **after** the Click-iT® detection reaction.
Fluorescent proteins such as Green Fluorescent Protein (GFP)	Use organic dye-based reagents, such as TC-FlAsH™ 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-FlAsH™ or TC-ReAsH™ reagents	Detect the tetracysteine (TC) tag with FlAsH™ or ReAsH™ reagents **before** the Click-iT® detection reaction.
Phalloidin	Phalloidin staining is **not compatible** with the Click-iT® 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® detection reaction.
R-phycoerythrin (R-PE) and R-PE-tandems such as Alexa Fluor® 680-R-PE	Use R-PE and R-PE tandems **after** the Click-iT® detection reaction.
Allophycocyanin (APC) and APC-tandems	**Completely compatible** with the Click-iT® detection reaction.

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

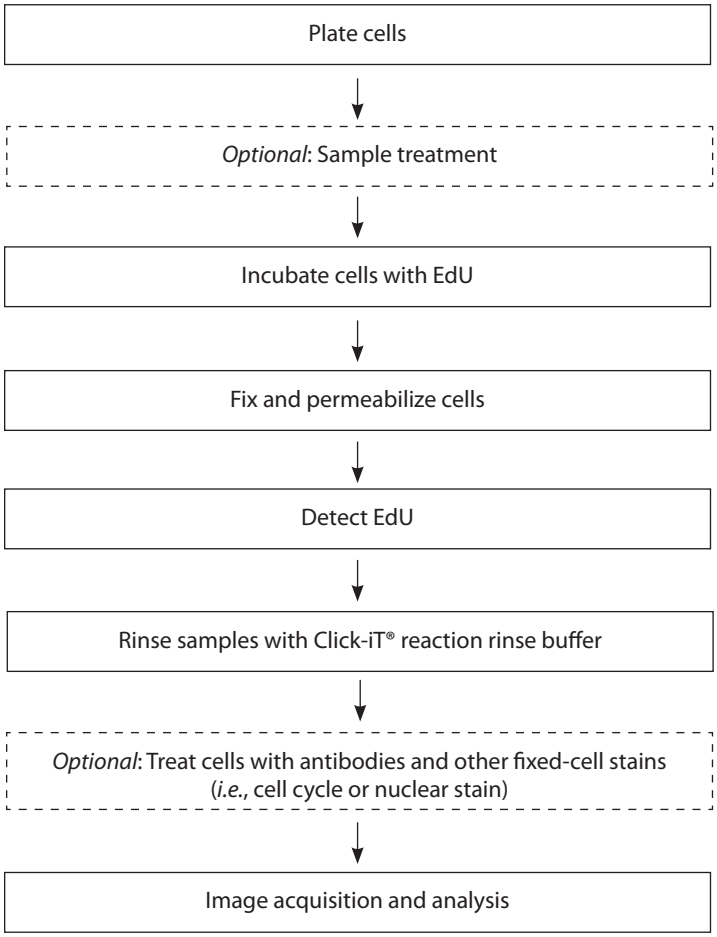

Figure 2. Workflow diagram for the Click-iT® EdU HCS Assay.

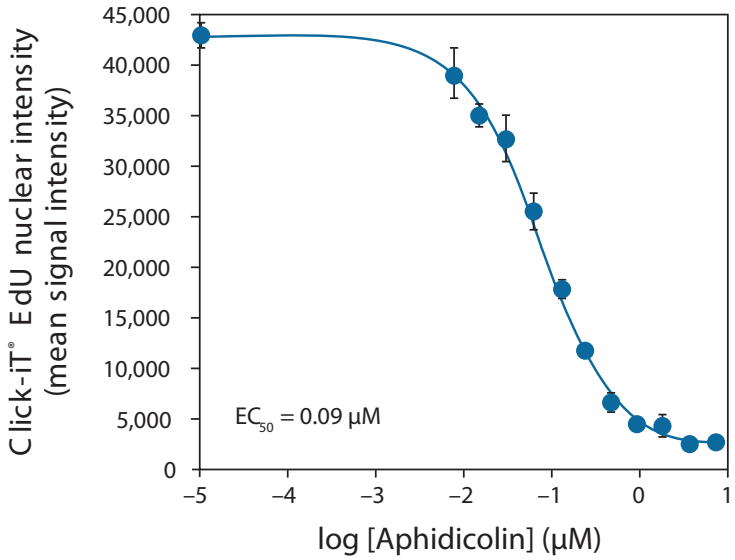

Figure 3. Dose response for aphidicolin in U-2 OS cells using the Click-iT® EdU Assay. U-2 OS cells were treated with the indicated amounts of aphidicolin for 3 hours, then its effects on DNA synthesis were assayed after a 1 hour incubation with 10 µM EdU. Cells were then fixed and permeabilized, and EdU incorporated into newly synthesized DNA was detected using the fluorescent Alexa Fluor® 555 azide from the Click-iT® EdU Alexa Fluor® 555 Assay. Quantitative analysis was performed using the Thermo Scientific Cellomics® ArrayScan® VTI and the Target Activation Bioapplication. The EC_{50} value was calculated using total EdU nuclear intensity which is plotted against various doses of aphidicolin.

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., 0.1% Triton® X-100 in PBS)
- Deionized water
- 96-well plates (as recommended for the specific imaging instrument)

Cautions

HCS NuclearMask™ Blue stain (Component G) is a mutagen. Use the dye with appropriate precautions.

Preparing Stock Solutions

1.1 To make a 10X stock solution of the Click-iT® EdU buffer additive (Component E), add 2 mL of deionized water to the vial and 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.

1.2 Prepare a working solution of 1X Click-iT® EdU reaction buffer (Component C) as follows:

For 2-plate kit (Cat. nos. C10350, C10352, C10354, C10356)

Prepare 40 mL of 1X Click-iT® EdU reaction buffer by transferring all of the solution (4 mL) in the Component C bottle to 36 mL of deionized water. Rinse the Component C bottle with some of the diluted Click-iT® EdU reaction buffer to ensure the transfer of all of the 10X concentrate.

For 10-plate kit (Cat. nos. C10351, C10353, C10355, C10357)

Prepare 150 mL of 1X Click-iT® EdU reaction buffer by transferring all of the solution (15 mL) in the Component C bottle to 135 mL of deionized water. Rinse the Component C bottle with some of the diluted Click-iT® EdU reaction buffer to ensure the transfer of all of the 10X concentrate.

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

Experimental Protocols

Labeling Cells with EdU

The following protocol was developed with A549, HeLa, and U-2 OS cells with an optimized EdU concentration of 10 µM, but can be adopted 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 EdU concentration for your cell type and experimental conditions. If currently using a BrdU assay for cell proliferation, a similar concentration to BrdU will be a good starting concentration for EdU.

2.1 Plate cells in 96-well plates at the desired density and allow cells to recover overnight at 37°C.

2.2 *Optional*: Treat cells with the compound of interest.

2.3 Prepare a 2X working solution of EdU (Component A) in complete medium from the 10 mM stock solution. Add 100 µL 2X EdU working solution to each well containing treated cells in 100 µL of complete medium, which results in 1X EdU (10 µM).

2.4 Incubate under normal cell culture conditions for the desired length of time. 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.

For longer incubations, i.e., >24 hours, we recommend lower concentrations of EdU. For shorter incubations, i.e., ≤ 30–45 minutes, higher concentrations could be used. For a negative staining control, include cells from the same population, but do not treat with EdU.

2.5 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

This protocol is optimized with a fixation step using 3.7% formaldehyde in PBS followed by a 0.1% Triton® X-100 permeabilization step, but is amenable to other fixation/permeabilization reagents such as methanol and saponin.

3.1 After EdU incubation, remove media and add 100 µL of 3.7% formaldehyde in PBS to each well. Incubate for 15 minutes at room temperature.

3.2 Remove fixative and wash each well twice with PBS.

Note: You can safely store the samples at this time.

3.3 Remove the wash solution. Add 100 µL of 0.1% Triton® X-100 in PBS to each well and incubate for 15 minutes at room temperature.

EdU Detection

4.1 Prepare 1X Click-iT® EdU buffer additive by diluting the 10X solution (prepared in step 1.1) 1:10 in deionized water. Prepare only as much of this solution as necessary for that day's experiments, and use on the same day. See Table 3 for details.

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

Note: Use the Click-iT® reaction cocktail immediately after preparation. The Click-iT® reaction buffer additive is susceptible to oxidation and is the limiting factor to the Click-iT® reaction cocktail's effectiveness over time.

Table 3. Click-iT® reaction cocktails.

Reaction components	Number of plates				
	0.5	1	2	5	10
1X Click-iT® EdU reaction buffer (prepared in step 1.2)	5.1 mL	10.3 mL	20.6 mL	51.5 mL	103 mL
$CuSO_4$ (Component D)	240 µL	480 µL	960 µL	2.4 mL	4.8 mL
Alexa Fluor® azide (Component B)	15 µL	30 µL	60 µL	150 µL	300 µL
1X Click-iT® EdU buffer additive (prepared in step 4.1)	600 µL	1.2 mL	2.4 mL	6 mL	12 mL
Total volume	6 mL	12 mL	24 mL	60 mL	120 mL

4.3 Remove the permeabilization buffer (step 3.3) and wash each well twice with PBS.

4.4 Remove wash solution and add 100 µL of Click-iT® reaction cocktail (prepared in step 4.2) to each well.

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

4.6 Remove the reaction cocktail and wash cells in each well once with 100 µL of Click-iT® reaction rinse buffer (Component F). Then remove the Click-iT®reaction rinse buffer.

4.7 *Optional*: Perform antibody labeling on 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.

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

DNA Staining

The following protocol is based upon 50 µL of HCS NuclearMask™ Blue working solution per well.

5.1 Dilute HCS NuclearMask™ Blue stain (Component G) solution 1:2,000 in PBS to obtain a 1X NuclearMask™ Blue stain working solution.

5.2 Remove any wash solution from cells.

5.3 Add 100 µL of 1X HCS NuclearMask™ Blue stain solution (prepared in step 5.1) to each well. Incubate for 30 minutes at room temperature, **protected from light**.

5.4 Remove the HCS NuclearMask™ Blue stain solution and wash each well twice with PBS. Remove the wash solution and proceed to **Imaging and Analysis**.

Imaging and Analysis

6.1 Add PBS to each well. Seal the plate with sealing film, if desired.

6.2 Scan the plate using an automated imaging platform equipped with filters appropriate for DAPI/Hoechst and FITC, Texas Red®, or Cy™5 dyes, depending on the Alexa Fluor® azide used. The nuclear region is characterized by the HCS NuclearMask™ Blue signal in the Hoechst channel. You may assess nascent DNA by determining signal intensity in the nuclear region in the FITC channel (if using Alexa Fluor® 488-labeled EdU, Cat. nos. C10350, C10351), the TRITC channel (if using Alexa Fluor® 555-labeled EdU, Cat. nos. C10352, C10353), the Texas Red® channel (if using Alexa Fluor® 594-labeled EdU, Cat. nos. C10354, C10355), or the Cy®5 channel (if using Alexa Fluor® 647-labeled EdU, Cat. nos. C10356, C10357).

When using the Thermo Scientific Cellomics® ArrayScan® VTI platform, use the Target Activation BioApplication. In channel 1, define the nuclear region with HCS NuclearMask™ Blue signal (the segmentation tool) as objects with Hoechst/XF93 filters. In channel 2, assess the nuclear spot fluorescence intensity of Alexa Fluor® 488-, 555-, 594, or 647-labeled EdU with the FITC/XF93, the TRITC/XF93, the Texas Red®/XF53, or the Cy®5/XF93 filters, respectively.

See Table 4 for the approximate fluorescence excitation/emission maxima for Alexa Fluor® dyes and HCS NuclearMask™ Blue stain bound to DNA.

Table 4. Approximate fluorescence excitation/emission maxima.

Fluorophore	Excitation (nm)	Emission (nm)
Alexa Fluor® 488	495	519
Alexa Fluor® 555	555	565
Alexa Fluor® 594	590	615
Alexa Fluor® 647	650	670
HCS NuclearMask™ Blue stain, bound to DNA	350	461

References

1. Proc Natl Acad Sci USA 105, 2415 (2008); **2.** Chem Bio Chem 4, 1147 (2003); **3.** J Am Chem Soc 124, 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
C10350	Click-iT® EdU Alexa Fluor® 488 HCS Assay *2-plate size*	1 kit
C10351	Click-iT® EdU Alexa Fluor® 488 HCS Assay *10-plate size*	1 kit
C10352	Click-iT® EdU Alexa Fluor® 555 HCS Assay *2-plate size*	1 kit
C10353	Click-iT® EdU Alexa Fluor® 555 HCS Assay *10-plate size*	1 kit
C10354	Click-iT® EdU Alexa Fluor® 594 HCS Assay *2-plate size*	1 kit
C10355	Click-iT® EdU Alexa Fluor® 594 HCS Assay *10-plate size*	1 kit
C10356	Click-iT® EdU Alexa Fluor® 647 HCS Assay *2-plate size*	1 kit
C10357	Click-iT® EdU Alexa Fluor® 647 HCS Assay *10-plate size*	1 kit
Related Products		
C10289	Click-iT® AHA Alexa Fluor® 488 Protein Synthesis HCS Assay	1 kit
C10327	Click-iT® RNA Alexa Fluor® 488 HCS Assay *2-plate size*	1 kit
C10328	Click-iT® RNA Alexa Fluor® 594 HCS Assay *2-plate size*	1 kit
C10337	Click-iT® EdU Alexa Fluor® 488 Imaging Kit *for 50 coverslips*	1 kit
C10338	Click-iT® EdU Alexa Fluor® 555 Imaging Kit *for 50 coverslips*	1 kit
C10339	Click-iT® EdU Alexa Fluor® 594 Imaging Kit *for 50 coverslips*	1 kit
C10340	Click-iT® EdU Alexa Fluor® 647 Imaging Kit *for 50 coverslips*	1 kit
H10294	HCS NuclearMask™ Deep Red stain *250X concentrate in DMSO*	400 µL
H10325	HCS NuclearMask™ Blue stain *for 10 × 96-well plates* *2000X concentrate*	65 µL
H10326	HCS NuclearMask™ Red stain *for 10 × 96-well plates* *1000X concentrate*	125 µL

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