Click-iT™ EdU Alexa Fluor® High-Throughput Imaging (HCS) Assay

Table 1. Contents and storage information.

Material	A10027	A10028	A10209	C10082	A10208	C10081	Concentration	Storage*
EdU (Component A)	255 µg	1.28 mg	255 µg	1.28 mg	255 µg	1.28 mg	NA	• 2–6˚C • Desiccate • Protect from light • DO NOT FREEZE
Alexa Fluor® azide (Component B)	1 vial (Alexa Fluor® 488)	1 vial (Alexa Fluor® 488)	1 vial (Alexa Fluor® 594)	1 vial (Alexa Fluor® 594)	1 vial (Alexa Fluor® 647)	1 vial (Alexa Fluor® 647)	NA	
Dimethylsulfoxide (DMSO, Component C)	500 µL	1 mL	500 µL	1 mL	500 µL	1 mL	NA	
Click-iT™ EdU reaction buffer (Component D)	4 mL	15 mL	4 mL	15 mL	4 mL	15 mL	10X solution containing Tris-buffered saline	
$CuSO_4$ (Component E)	1 vial	1 vial	1 vial	1 vial	1 vial	1 vial	100 mM aqueous solution	
Click-iT™ EdU buffer additive (Component F)	400 mg	400 mg	400 mg	400 mg	400 mg	400 mg	NA	
Hoechst 33342 (Component G)	25 µL	125 µL	25 µL	125 µL	25 µL	125 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.

Stability: When stored as directed this kit is stable for 1 year.

Number of assays: Sufficient material is supplied for 2 × 96 tests (Cat. nos. A10027, A10208, A10209) or 10 × 96 tests (Cat. nos. A10028, C10081, C10082), 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., ^{3}H-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^{+1}) 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 kit is available in a 2-plate and 10-plate size, and includes sufficient reagents for performing 2 × 96 or 10 × 96 tests, respectively, in a plate-based assay format using 100 µL reaction buffer per test.

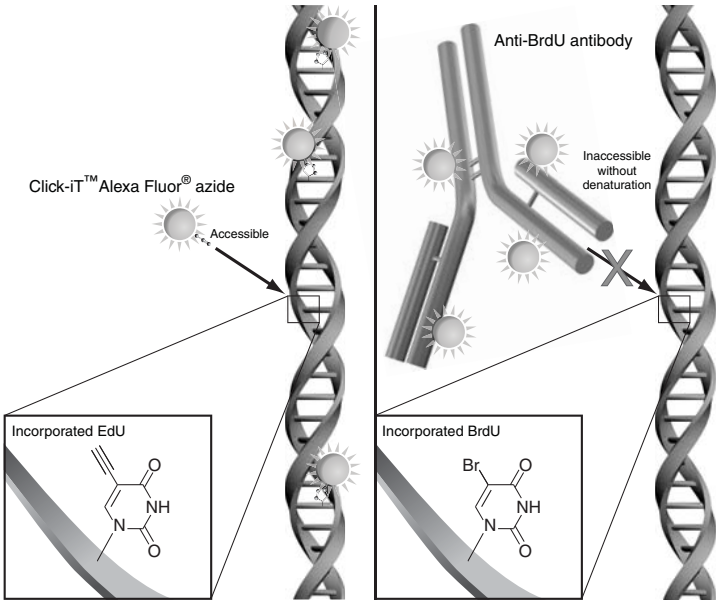

Figure 1. Detection of the incorporated EdU with the Alexa Fluor® azide versus incorporated BrdU with an anti-BrdU antibody. The small size of the Alexa Fluor® azide eliminates the need to denature the DNA for the EdU detection reagent to gain access to the nucleotide.

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-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.

*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.

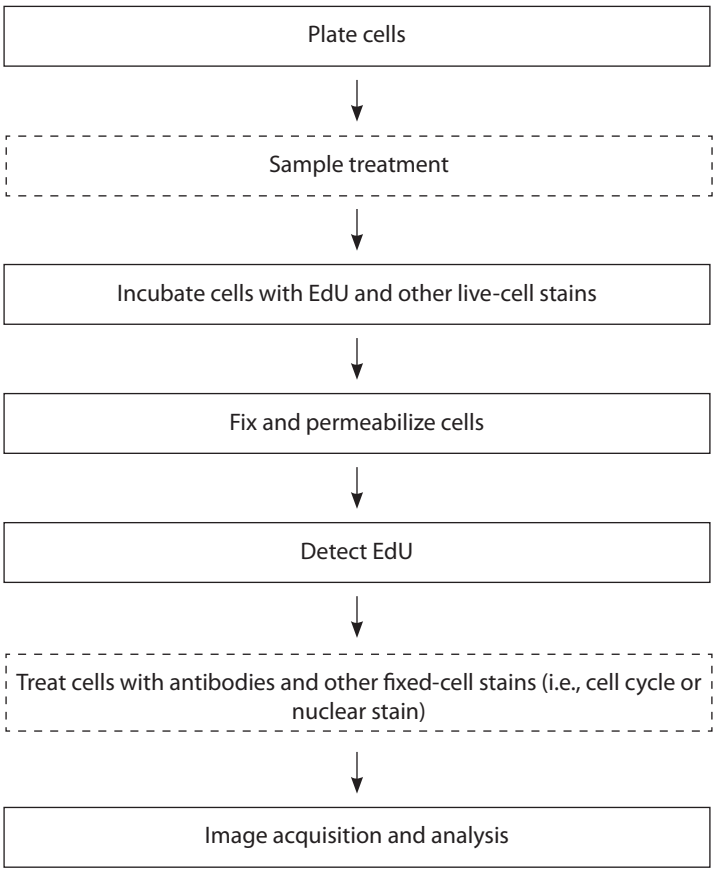

Figure 2. Workflow diagram for the Click-iT™ EdU Cell Proliferation Assay for High-Throughput imaging (HCS).

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
- 96-well plates (recommended for the specific imaging instrument)

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) as follows:

- For 2-plate kit (Cat. nos. A10027, A10208, A10209), add 100 µL DMSO (Component C) or aqueous solution (i.e. buffer, saline) to EdU (Component A) and mix well.
- For 10-plate kit (Cat. nos. A10028, C10081, C10082), add 500 µL 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 ≤–20˚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) as follows:

- For 2-plate kit (Cat. nos. A10027, A10208, A10209), add 70 µL DMSO (Component C) to Component B and mix well.
- For 10-plate kit (Cat. nos. A10028, C10081, C10082), add 330 µL DMSO (Component C) to Component B and mix well.

After use, store any remaining working solution at ≤–20˚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) as follows:

- For 2-plate kit (Cat. nos. A10027, A10208, A10209), make 40 mL 1X Click-iT™ EdU reaction buffer 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.
- For 10-plate kit (Cat. nos. A10028, C10081, C10082), make 150 mL of 1X Click-iT™ EdU reaction buffer by transferring all of the solution (15 mL) in the Component D bottle to 135 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˚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 ≤–20˚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 in 96-well plates at desired density and allow cells to recover overnight before additional treatment.

2.2 Treat the cells as desired.

2.3 Prepare a 2X working solution of EdU (Component A) in complete medium from the 10 mM stock solution prepared in step 1.2. A suggested starting concentration range is 10–20 µM. Add 100 µL 2X EdU stock solution to each well containing 100 µL of complete medium.

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

2.4 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.

2.5 Proceed immediately to **Cell Fixation and Permeabilization** (steps 3.1–3.4) 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.

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

3.2 Remove fixative and wash cells twice with 100 µL/well 3% BSA in PBS.

3.3 Remove the wash solution.

3.4 Add 100 µL/well 0.5% Triton® X-100 in PBS 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.

4.3 Remove the permeabilization buffer (step 3.4) and wash cells twice with 100 µL/well 3% BSA in PBS. Remove the wash solution.

4.4 Add 100 µL Click-iT™ reaction cocktail (prepared in step 4.2) to each well and mix well.

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

4.6 Remove the reaction cocktail and wash once with 100 µL /well 3% BSA in PBS. Remove the wash solution.

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

Table 3. Click-iT™ reaction cocktails.

Reaction components	Number of plates						
	0.5	1	2	3	4	5	10
1X Click-iT™ reaction buffer (prepared in step 1.4)	5.1 mL	10.3 mL	20.6 mL	30.9 mL	41.2 mL	51.5 mL	103 mL
$CuSO_4$ (Component H)	240 µL	480 µL	960 µL	1.44 mL	1.92 mL	2.4 mL	4.8 mL
Alexa Fluor® azide (prepared in step 1.3)	15 µL	30 µL	60 µL	90 µL	120 µL	150 µL	300 µL
Reaction buffer additive (prepared in step 4.1)	600 µL	1.2 mL	2.4 mL	3.6 mL	4.8 mL	6.0 mL	12 mL
Total volume	6 mL	12 mL	24 mL	36 mL	48 mL	60 mL	120 mL

Antibody Detection (optional)

5.1 If required, block the plate with 100 µL/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 at least 40 µL/well of 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 100 µL/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 100 µL/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 and Cell Cycle Distribution

6.1 Wash each well with 100 µL/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 100 µL/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 100 µL/well PBS. Remove the wash solution.

Imaging and Analysis

7.1 Add 100 µL/well PBS. Seal the plate with plate sealing film, if desired.

7.2 Image and analyze the plate using instrument filter sets appropriate for Hoechst 33342, the Alexa Fluor® dye, and any secondary reagent used in the experiment.

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
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
A10209	Click-iT™ EdU Alexa Fluor® 594 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
A10208	Click-iT™ EdU Alexa Fluor® 647 High-Throughput Imaging (HCS) Assay *2-plate size*	1 kit
C10081	Click-iT™ EdU Alexa Fluor® 647 High-Throughput Imaging (HCS) Assay *10-plate size*	1 kit
Related Products		
A10034	Click-iT™ EdU Pacific Blue™ Flow Cytometry Assay Kit *50 assays*	1 kit
A10044	EdU (5-ethynyl-2´-deoxyuridine)	50 mg
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
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
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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