Click-iT® EdU Microplate Assay

Catalog no. C10214

Table 1. Contents and storage information.

Material	Amount	Concentration	Storage*	Stability
EdU (Component A)	50 µL	10 mM in DMSO	• –20˚C • Desiccate • Protect from light	When stored as directed this kit is stable for 1 year
Oregon Green® 488 azide (Component B)	150 µL	NA		
Dimethylsulfoxide (DMSO, Component C)	100 µL	NA		
Click-iT® EdU fixative (Component D)	30 mL	NA		
Click-iT® EdU reaction buffer (Component E)	3 mL	10X concentrate		
$CuSO_4$ (Component F)	1 mL	100 mM aqueous solution		
Click-iT® EdU buffer additive (Component G)	1 mL	NA		
Blocking buffer (Component H)	125 mL	2X concentrate		
Anti-Oregon Green® rabbit IgG, horseradish peroxidase (HRP) conjugate (Component I)	1 vial	NA		
Amplex® UltraRed buffer (Component J)	25 mL	10X concentrate		
Amplex® UltraRed reagent (Component K)	1 vial	NA		
Hydrogen peroxide (H_2O_2, Component L)	200 µL	stabilized 3% solution		
Amplex® UltraRed stop reagent (Component M)	1 vial	NA		

*These storage conditions are appropriate when storing the entire kit upon receipt. After preparing stock solutions, optimal storage may change. For storing prepared stock solutions, follow recommendations included in this product information sheet. NA = Not applicable.

Number of assays: Sufficient material is supplied for 400 assays based on the protocol below.

Approximate fluorescence excitation/emission maxima: Oregon Green® 488 azide**:** 495/519 in nm; Amplex® UltraRed reagent: 568/585 in nm.

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 the radioactive nucleoside, ^{3}H-thymidine but was later replaced by antibody-based detection of the nucleoside analog bromo-deoxyuridine (BrdU). The Click-iT® EdU Microplate Assay from Invitrogen is a novel alternative to the BrdU assay and uses the nucleoside analog EdU (5-ethynyl-2´-deoxyuridine) which is incorporated into DNA during active DNA synthesis.

The EdU and BrdU methods differ in the detection method; EdU detection is based on a click reaction—a copper-catalyzed covalent reaction between an azide and an alkyne.[1-5] In this application, the EdU contains the alkyne and Oregon Green® 488 dye contains the azide. The advantages of the Click-iT® EdU labeling are readily evident while performing the assay. The small size of the green-fluorescent Oregon Green® 488 azide (MW ~638) as compared to an anti-BrdU antibody (MW ~150,000) allows for efficient detection of the incorporated EdU using mild conditions and does not require DNA denaturation (Figure 1).

The Click-iT® EdU microplate assay has been successfully tested in HeLa, A549, U-2 OS, and A541 cells with a variety of reagents that modulate DNA synthesis including the DNA synthesis inhibitor, aphidicolin, and the mitotic inhibitor, paclitaxel (Figure 2). The kit contains sufficient reagents for performing 400 individual assays in a 96-well plate format.

Click-iT® EdU Microplate Assay

The Click-iT® EdU microplate assay protocol differs from the Click-iT® EdU assays for fluorescence microscopy, flow cytometry, or high-throughput imaging. The EdU microplate assay requires signal amplification to achieve a suitable sensitivity limit and dynamic range.

After labeling the cells with EdU, the cells are fixed, and click labeling reaction containing copper and Oregon Green® 488 azide is performed. The hapten, Oregon Green® 488, is used instead of biotin to eliminate biotin blocking steps required by cells possessing high levels of endogenous biotin. At this point, the cells can be analyzed by fluorescence microscopy or high-throughput imaging using filters optimized for fluorescein or the Alexa Fluor® 488 dye. Although this is not a required step in the protocol, it is useful to verify if there was any loss of cells or whether the cells have incorporated the EdU before proceeding with the signal amplification steps. The signal amplification steps include incubation with an anti-Oregon Green® antibody conjugated to the enzyme, horseradish peroxidase (HRP), which then reacts with Amplex® UltraRed substrate in a 1:1 stoichiometry and produces a brightly red, fluorescent product (excitation/emission maxima ~568/585 nm, Figure 3).

With a high extinction coefficient, good quantum efficiency, and resistance to autooxidation, the fluorescence-based Amplex® UltraRed reagent delivers higher sensitivity and a broader assay range than other fluorogenic or colorimetric peroxidase substrates. The kit also includes the Amplex® UltraRed stop reagent that enables the peroxidase reaction to be terminated at a user-determined time point and stabilizes the signal for up to 24 hours.

Before You Begin

Materials Required but Not Provided

- 95% ethanol
- Deionized water or 18 megohm purified water
- Phosphate buffered-saline (PBS, Invitrogen Cat. no. 14190-144 or 14190-250)
- 96-well plates (recommended for the specific plate reader or cell type)

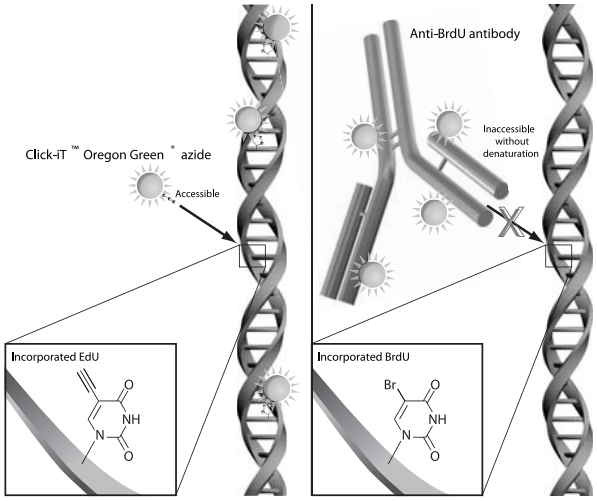

Figure 1. Detection of the incorporated EdU with the Oregon Green® 488 azide versus incorporated BrdU with an anti-BrdU antibody.

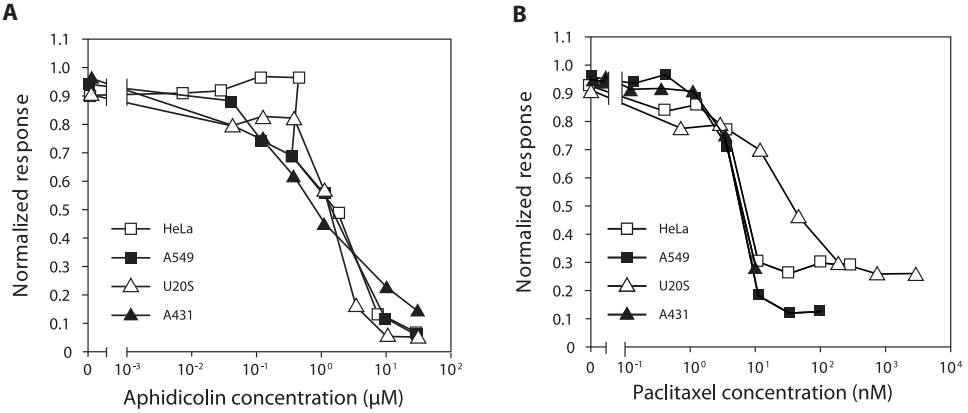

Figure 2. Detection of proliferation using the Click-iT® EdU microplate assay with aphidicolin or paclitaxel treated cells. HeLa, A549, U-2 OS, and A431 cells were all treated with serial dilution of (A) aphidicolin, a DNA synthesis inhibitor or (B) paclitaxel, a mitotic inhibitor, for 24 hours and pulsed with 10 μM EdU for 2 hours prior to fixation, and click labeling.

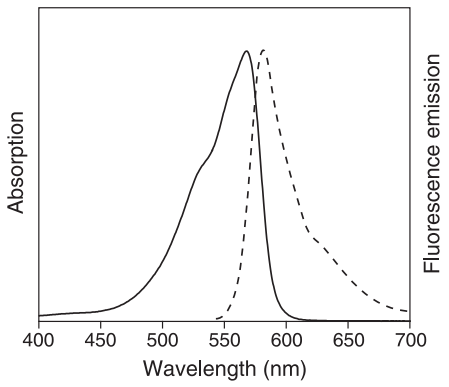

Figure 3. Normalized absorption and fluorescence emission spectra for the Amplex® UltraRed product.

Caution 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 Stock Solutions

1.1 Allow the vials to completely thaw and warm to room temperature before opening.

Note: Because of the large vial size, several hours may be needed to thaw the vials.

1.2 Prepare a working solution of 2X Click-iT® EdU reaction buffer (Component E) by transferring all of the solution (3 mL) in the Component E bottle to 27 mL of deionized water. Rinse the Component E 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 the Click-iT® reaction buffer, dilute volumes from the Component E bottle 1:10 with deionized water.

After use, store any remaining stock solution at 4˚C or at room temperature. When stored as directed, this stock solution is stable for up to 1 year.

1.3 Prepare a **fresh** working solution of Click-iT® EdU buffer additive (Component G) by transferring all of the solution (1 mL) in the Component G bottle to 9 mL of deionized water. To make smaller amounts of the Click-iT® EdU buffer additive, dilute volumes from the Component G bottle 1:10 with deionized water. Refer to Table 2 for the amount of additive required. Use the working solution on the same day.

1.4 Prepare a 1X working solution of blocking buffer (Component H) by transferring all of the solution (125 mL) in the Component H bottle to 125 mL of deionized water. To make smaller amounts of the blocking buffer, dilute volumes from the Component H bottle 1:2 with deionized water.

After use, store any remaining solution at 4° C. When stored as directed, this solution is stable for up to one month. **Do not** add sodium azide as a preservative.

1.5 Prepare a stock solution of the anti-Oregon Green® HRP conjugate (Component I) by adding 75 µL of deionized water to the vial. Mix by gentle pipetting or by inversion to avoid foaming. **Do not vortex.** Centrifuge antibody conjugate briefly prior to use.

After use, store any remaining stock solution at 2–6˚C (short-term) or for longer periods with the addition of 0.02% thimerosal. **Note: Do not** use sodium azide with peroxidase enzyme conjugates. When stored as directed, this stock solution is stable for up to 6 months.

1.6 Prepare a working solution of the Amplex® UltraRed buffer (Component J) by transferring all of the solution (25 mL) in the Component J bottle to 225 mL of deionized water. To make smaller amounts of reaction buffer, dilute volumes from the Component J bottle 1:10 with deionized water.

After use, store any remaining stock solution at 4˚C. When stored as directed, this stock solution is stable for up to 1 year.

1.7 Prepare the stock solution of the Amplex® UltraRed reagent (Component K) by adding 68 µL DMSO (Component C) to the Component K vial and mix well.

After use, store any remaining stock solution at –20˚C. When stored as directed, this stock solution is stable for up to 1 year.

1.8 Prepare **fresh** hydrogen peroxide stock solution by adding 4 µL of Component L to 70 µL of deionized water. Use this solution on the same day.

1.9 To make a stock solution of Amplex® UltraRed stop reagent (Component M), add 5.8 mL of 95% ethanol to the bottle. Vortex vigorously until the solid is completely 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, A431, U-2 OS, and M1WT3 cells with an optimized EdU concentration of 10 µM, but can be adapted for any cell type. Growth medium, cell density, cell type variations, and other factors may influence signal. 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 supplied for standard dose response, additional EdU is available separately from Invitrogen (Cat. no. A10044). If currently using a BrdU based assay for cell proliferation, a similar concentration to BrdU will be a good starting concentration for EdU. A workflow diagram for the Click-iT® EdU microplate assay (Figure 4) is included on the next page.

2.1 Plate cells at a desired density and allow cells to recover overnight before additional treatment.

2.2 Treat as desired.

2.3 Prepare a working stock of EdU (Component A) in prewarmed complete media. A suggested starting concentration range is 10–20 µM, similar to BrdU. For longer incubations, i.e. >24 hours, lower concentrations are recommended.

To make 10 µM EdU final concentration, make a 10X EdU working stock by diluting the 10 mM supplied stock solution 1:100 in complete media. Add 10 µl of the 10X EdU working stock to each well containing 100 µL of media.

Note: Place plates on a rotary shaker for 2 minutes to adequately disperse the EdU in the well.

2.4 Incubate under conditions optimal for cell type for 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 to **Cell Fixation and Click Labeling**.

Cell Fixation and Click Labeling

3.1 Prepare 2X Click-iT® reaction cocktail according to Table 2.

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

3.2 After incubation with EdU, remove media, then add 50 µL/well Click-iT® EdU Fixative (Component D) and incubate for 5 minutes at room temperature.

3.3 Add 50 µL of the 2X Click-iT® reaction cocktail (from step 3.1) directly to the wells containing 50 µL of Click-iT® EdU Fixative.

3.4 Incubate for 25 minutes at room temperature, **protected from light**.

3.5 Remove the reaction cocktail.

Table 2. 2X Click-iT® reaction cocktails.

Reaction components	Number of plates			
	1	2	3	4
2X Click-iT® reaction buffer (prepared in step 1.2)	4.7 mL	9.3 mL	14 mL	18.6 mL
$CuSO_4$ (Component F)	120 µL	240 µL	360 µL	480 µL
Oregon Green® 488 azide (Component B)	30 µL	60 µL	90 µL	120 µL
Click-iT® EdU buffer additive (prepared in step 1.3)	1.2 mL	2.4 mL	3.6 mL	4.8 mL
Total volume	6 mL	12 mL	18 mL	24 mL

3.6 Wash wells twice with 200 µL/well of 1X blocking buffer prepared in step 1.4. Proceed to **Antibody Incubation**.

Antibody Incubation

4.1 For 4 × 96-well plates, prepare a working solution of anti-Oregon Green® HRP conjugate (Component I) by diluting 60 µL antibody conjugate in 24 mL 1X blocking buffer prepared in step 1.4. Mix by gentle inversion. **Do not** vortex. To prepare a smaller volume, dilute anti-Oregon Green® HRP conjugate (Component I) 1:400 with the 1X blocking buffer.

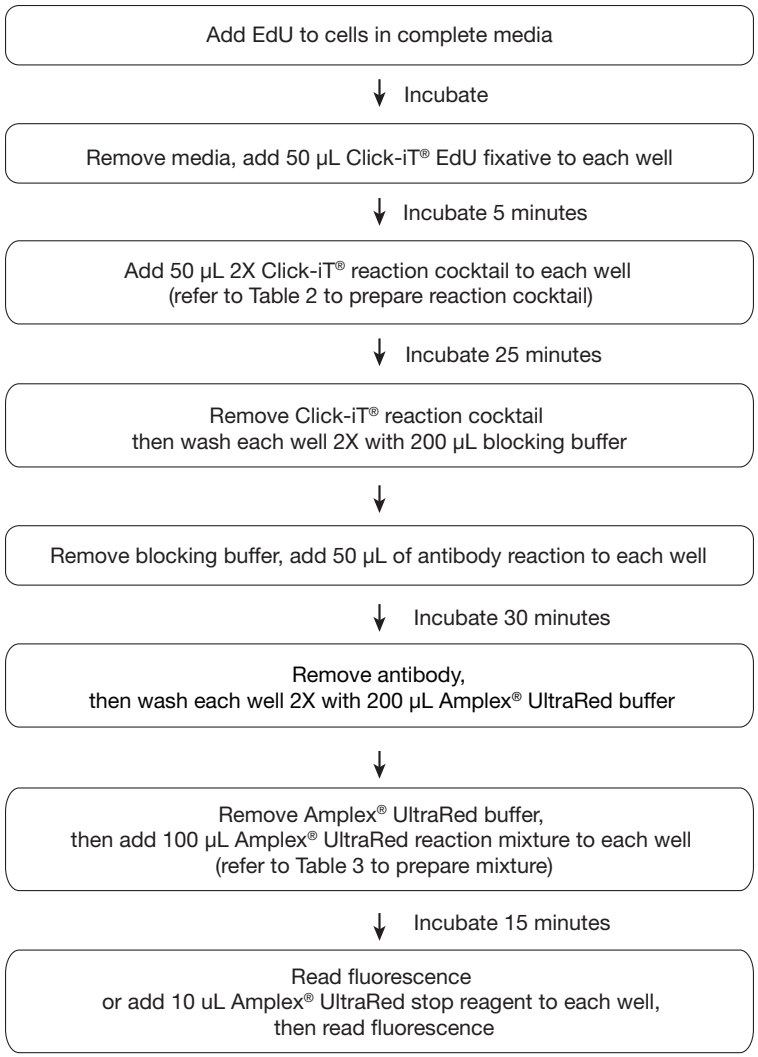

Figure 4. Workflow diagram for the Click-iT® EdU Microplate Assay.

4.2 Remove wash buffer.

4.3 Add 50 µL of the diluted anti-Oregon Green® HRP conjugate solution (from step 4.1) to each well.

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

4.5 Wash twice with 200 µL/well of Amplex® UltraRed buffer prepared in step 1.6. Proceed to **Amplex® UltraRed Detection**.

Amplex® UltraRed Detection

5.1 Prepare the Amplex® UltraRed reaction mixture according to Table 3.

5.2 Remove wash buffer from the wells.

5.3 Start the reaction by adding 100 µL/well of Amplex® UltraRed reaction mixture from step 5.1.

Note: For kinetic measurements, begin collecting data after the addition of the Amplex® UltraRed reagent

5.4 Incubate for 15 minutes at room temperature, protected from light.

5.5 Add 10 µL/well Amplex® UltraRed stop reagent (prepared in step 1.9).

Note: Pipetting volume using the ethanol based stop reagent is improved by increasing the dispense uptake to insure that the last wells have sufficient remaining volume to deliver.

5.6 Proceed to **Quantitation and Analysis**.

Table 3. Amplex® UltraRed reaction mixture.

Reaction components	Number of plates			
	1	2	3	4
Amplex® UltraRed buffer (prepared in step 1.6)	12 mL	24 mL	36 mL	48 mL
Amplex® UltraRed reagent (prepared in step 1.7)	12 µL	24 µL	36 µL	48 µL
Hydrogen peroxide (prepared in step 1.8)	12 µL	24 µL	36 µL	48 µL
Total volume	12 mL	24 mL	36 mL	48 mL

Quantitation and Analysis

Analyze the plate on a plate reader with filter sets appropriate for Amplex® UltraRed (Figure 3).

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). **5.** Anal Biochem 253, 162 (1997).

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

Cat. no.	Product Name	Unit Size
C10214	Click-iT® EdU Microplate assay *400 assays*	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
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
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
A33855	Amplex® Red/UltraRed stop reagent	1 set
A36006	Amplex® UltraRed reagent	5 × 1 mg
14190-144	Dulbecco's Phosphate-Buffered Saline (D-PBS) (1X), liquid	500 mL
14190-250	Dulbecco's Phosphate-Buffered Saline (D-PBS) (1X), liquid	10 × 500 mL

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