Click-iT™ EdU Proliferation Assay for Microplates

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WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Product description

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 to directly measure DNA synthesis was performed by incorporation of the radioactive nucleoside (e.g., ^{3}H-thymidine), however this method was replaced by the antibody-based detection of the nucleoside analog bromo-deoxyuridine (BrdU). After incorporation of the BrdU into newly synthesized DNA, denaturation is required to expose the BrdU molecules to the anti-BrdU antibody, but this denaturation requires harsh methods (HCl, heat, or enzymes) while also being inefficient and inconsistent.

The Invitrogen™ Click-iT™ EdU Proliferation Assay for Microplates is an alternative to the BrdU assay. The Click-iT™ assay uses the highly specific, copper-catalyzed covalent reaction between an azide and an alkyne group to join molecules. Click-iT™ technology uses the nucleoside analog EdU (5-ethynyl-2′-deoxyuridine), which is added to live cells and incorporated into DNA during active DNA synthesis. The incorporated EdU contains the alkyne group, which is covalently joined to the azide group present on HRP (horseradish peroxidase) by using click chemistry. After the covalent attachment, Amplex™ UltraRed reagent is added and the conversion to a highly fluorescent product is recorded by using a fluorescence microplate reader using an excitation of 568 nm and an emission of 585 nm (Figure 2). With a high extinction coefficient, good quantum efficiency, and resistance to auto-oxidation, Amplex™ UltraRed reagent delivers brighter fluorescence and higher sensitivity while also providing versatility through detection by either fluorescence or absorbance measurement.

Compared to anti-BrdU-based microplate proliferation assays, the Click-iT™ EdU Proliferation Assay for Microplates uses small reaction moieties and does not require antibodies or DNA denaturation, both of which can reduce assay performance (Figure 1). Additionally, streptavidin-biotin binding is not required, eliminating the necessary biotin blocking steps required by cells possessing high levels of endogenous biotin.

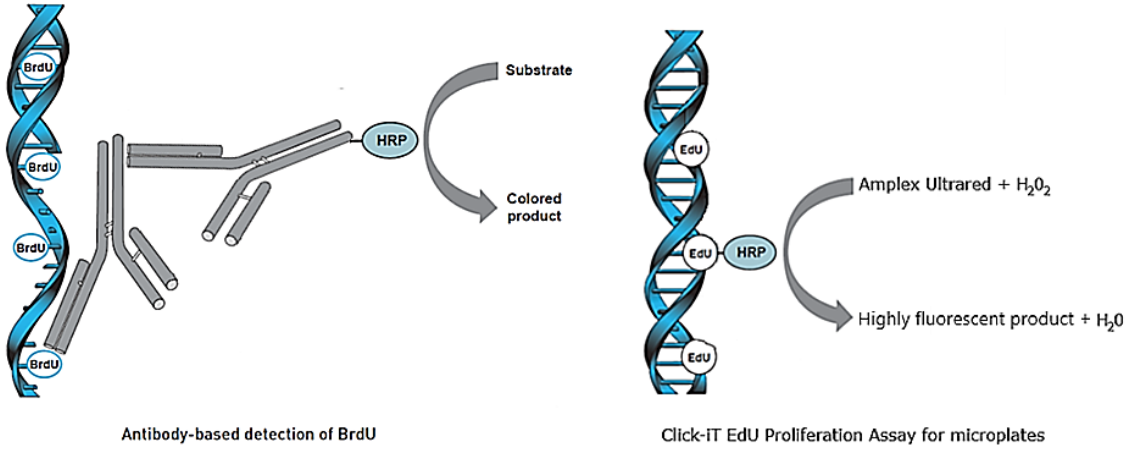

Fig. 1 Comparison of antibody-based BrdU and Click-iT™ EdU proliferation assays.

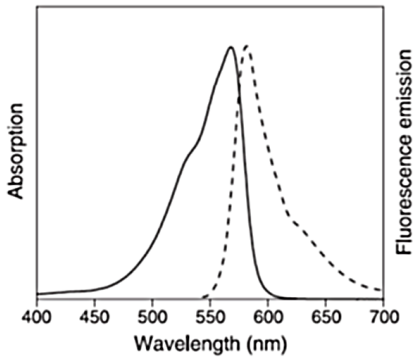

Fig. 2 Normalized excitation and emission spectra for the Amplex™ UltraRed product. Excitation maximum of 568 nm and emission maximum of 585 nm are recommended for detection.

Contents and storage

Upon receipt, store the kit at –20°C. When stored as directed, the product is stable for up to 6 months after receipt. Reagents provided in the kit are sufficient for 400 reactions in 96-well plates.

Contents	Quantity	Concentration	Storage
EdU (Component A)	50 µL	10 mM in DMSO	• –20°C • Desiccate • Protect from light
HRP-azide (Component B)	2 vials	—	
Dimethylsulfoxide (DMSO) (Component C)	100 µL	—	
Click-iT™ EdU fixative (Component D)	30 mL	—	
Click-iT™ EdU master mix (Component E)	5 mL	5.7X concentrate	
Click-iT™ EdU reaction additive (Component F)	1 mL	—	
Click-iT™ EdU wash buffer additive (Component G)	5 mL	200X concentrate	
Amplex™ UltraRed reaction buffer (Component H)	50 mL	10X concentrate	
Amplex™ UltraRed reagent (Component I)	1 mg	—	
Hydrogen peroxide (H_2O_2) (Component J)	200 µL	Stabilized 3% solution	
Amplex™ UltraRed stop reagent (Component K)	1 vial	—	

CAUTION! DMSO (Component C) solvent 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.

Required materials not provided

- 95% ethanol
- 1.5% solution of Bovine Serum Albumin (BSA) in PBS
- Deionized water
- Phosphate-buffered saline (PBS, Catalog No. 10010023)
- 96-well plates (recommended for the specific plate reader or cell type)

Workflow

Add EdU to cells in complete media.
Incubate.
▼
Remove media, add 50 µL Click-iT™ EdU fixative to each well.
Incubate 5 mins.
▼
Remove Click-iT™ EdU fixative, then wash each well.
▼
Add 50 µL 1X Click-iT™ reaction cocktail to each well.
(Refer to Table 1 to prepare reaction cocktail.)
Incubate 30 mins.
▼
Remove Click-iT™ reaction cocktail, then add 200 µL blocking buffer.
Incubate 5 mins.
▼
Remove blocking buffer, then wash each well 3 times.
▼
Add 100 µL Amplex™ UltraRed reaction mixture to each well.
(Refer to Table 2 to prepare mixture.)
Incubate 15 mins.
▼
Read fluorescence or add 10 µL Amplex™ UltraRed stop reagent to each well, then read fluorescence.

Prepare stock solutions

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

1. **HRP-azide stock solution** - For 1 or 2 plates, prepare a stock solution of the HRP-azide (Component B) by adding 55 µL of PBS to one vial. For 3 or 4 plates, prepare a stock solution of the HRP-azide (Component B) by adding 55 µL of PBS to both vials. Mix by gentle pipetting to avoid foaming. Do not vortex. After resuspension, invert and then centrifuge to ensure all material has been resuspended. After use, any remaining stock solution should be stored for short periods at 4°C. When stored as directed, this stock solution is stable for up to 3 months. **Do not re-freeze the HRP-azide solution**.
2. **Amplex™ UltraRed reagent** - Prepare the stock solution of the Amplex™ UltraRed reagent by adding 68 µL DMSO (Component C) to the Amplex™ UltraRed reagent (Component I) vial and mix well.

   Note: After use, store any remaining stock solution at -20°C. When stored as directed, this stock solution is stable for up to 1 year.
3. **Click-iT™ EdU reaction additive** - The Click-iT™ EdU reaction additive needs to be made **fresh** and used on the same day. Refer to Table 1 for the amount of Click-iT™ EdU reaction additive (Component F) required for the number of 96-well plates being tested. Based on amount needed perform a 1:10 dilution of the Click-iT™ EdU reaction additive (Component F) into deionized water.
4. **Click-iT™ wash buffer** - For each 96-well plate being tested, prepare 1X Click-iT™ EdU wash buffer by adding 0.5 mL of the 200X Click-iT™ wash buffer additive (Component G) into 99.5 mL of PBS. The resulting of amount of 1X Click-iT™ EdU wash buffer is sufficient for one 96-well plate.
5. **Amplex™ UltraRed reaction buffer** - Refer to Table 2 for the amount of Amplex™ UltraRed reaction buffer (Component H) that is required for your experiment. To make the required amount dilute the Amplex™ UltraRed reaction buffer (Component H) 1:10 with deionized water.

   Note: After use, store any remaining stock solution at 4°C. When stored as directed, this stock solution is stable for up to 1 year.
6. **Hydrogen peroxide solution** - Prepare fresh hydrogen peroxide stock solution by adding 4 µL of Hydrogen peroxide (Component J) to 70 µL of deionized water.

IMPORTANT! Use this solution on the same day it is prepared.

7. **Amplex™ UltraRed stop reagent** - To make a stock solution of Amplex™ UltraRed stop reagent, add 5.8 mL of 95% ethanol to the Amplex™ UltraRed stop reagent (Component K) bottle. Vortex vigorously. If the reagent is not completely in solution, incubate at room temperature until dissolved.

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

Label cells with Click-iT™ EdU

- The following protocol was developed using A549, HeLa and U-2 OS cells with an optimized EdU concentration of 10 µM. However, the kit can be adapted to any cell type.
- Growth medium, cell density, cell-type variations, and other factors may influence signal. For 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 (Thermo Fisher Scientific™, Cat. No. A10044).
- A BrdU-based assay for cell proliferation can provide a good starting concentration, as BrdU acts similarly to EdU.

1. Plate cells at a desired density and incubate overnight.
2. Treat as desired.
3. Prepare a working stock of EdU (Component A) in pre-warmed 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 5X EdU working stock by diluting the 10 mM supplied stock solution 1:200 into pre-warmed complete media. Add 25 µL of the 5X EdU working stock to each well containing 100 µL of media. Mix to adequately disperse the EdU in the well.
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.

Fix and click label cells

1. After incubation with EdU, remove media, add 50 µL/well of the Click-iT™ EdU fixative (Component D) and incubate for 5 minutes at room temperature.
2. Prepare the 1X Click-iT™ reaction cocktail (see Table 1) no more than 15 minutes prior to use.

   Table 1 Click-iT™ reaction cocktail.

Reaction Cocktail Components[1]	Number of Plates			
	1	2	3	4
Deionized water	4.3 mL	8.6 mL	12.9 mL	17.2 mL
Click-iT™ EdU master mix (Component E)	1.1 mL	2.2 mL	3.2 mL	4.3 mL
HRP-azide (step 1 on page 2)	25 µL	50 µL	75 µL	100 µL
Click-iT™ EdU reaction additive (step 3 on page 2)	600 µL	1.2 mL	1.8 mL	2.4 mL

[1] Reagents are prepared in "Prepare stock solutions" on page 2.

3. Remove Click-iT™ EdU fixative, invert over paper towel, and tap dry.
4. Add 200 µL/well of 1X Click-iT™ EdU wash buffer (step 4 on page 2), invert over paper towel, and tap dry.
5. Add 50 µL of the 1X Click-iT™ EdU reaction cocktail (Table 1) per well and incubate for 30 minutes at room temperature.
6. Remove the Click-iT™ EdU reaction cocktail, invert over paper towel, and tap dry.
7. Add 200 µL of 1.5% BSA Blocking Solution to each well and incubate for 5 minutes at room temperature protected from light.
8. Prepare the Amplex™ UltraRed reaction mixture according to this table.

   Table 2 Amplex™ UltraRed reaction mixture.

Reaction Mixture Components[1]	Number of Plates			
	1	2	3	4
Amplex™ UltraRed Reaction Buffer (step 5 on page 2)	12 mL	24 mL	36 mL	48 mL
Amplex™ UltraRed Reagent (step 2 on page 2)	12 µL	24 µL	36 µL	48 µL
Hydrogen peroxide solution (step 6 on page 2)	12 µL	24 µL	36 µL	48 µL

[1] Reagents are prepared in "Prepare stock solutions" on page 2.

9. Remove the 1.5% BSA Blocking Solution, invert over a paper towel, and tap dry.
10. Wash 3 times with 200 µL/well of 1X Click-iT™ EdU wash buffer (step 4 on page 2). Between each wash, invert over a paper towel and tap dry.

    Note: Do not allow the wells to dry. Proceed immediately to the next step and add the Amplex™ UltraRed reaction mixture.
11. Start the reaction by adding 100 µL of the Amplex™ UltraRed reaction mixture prepared in Table 2 to each of the wells and incubate for 15 minutes at room temperature, protected from light.
12. Stop the reaction by adding 10 µL/well of Amplex™ UltraRed stop solution (step 7 on page 3), then mix the wells to ensure the reaction has stopped.
13. Read on a microplate reader using filter sets appropriate (excitation 568 nm and emission 585 nm) for Amplex™ UltraRed reagent (Figure 2).

Limited product warranty

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