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 is by directly measuring DNA synthesis. Initially, this was performed by incorporation of radioactive nucleosides, i.e., 3H-thymidine. This method was replaced by antibodybased detection of the nucleoside analog bromo-deoxyuridine (BrdU). The Click-iT® EdU Flow Cytometry Assay Kits are novel alternatives to the BrdU assay. EdU (5-ethynyl-2´-deoxyuridine) is a nucleoside analog to thymidine and is incorporated into DNA during active DNA synthesis. Detection is based on a click reaction (1-4), a copper catalyzed covalent reaction between an azide and an alkyne. In this application, the alkyne is found in the ethynyl moiety of EdU, while the azide is coupled to Pacific Blue™ dye, Alexa Fluor® 647 dye, or Alexa Fluor® 488 dye. Standard flow cytometry methods are used for determining the percentage of S-phase cells in the population (Figure 1).

The advantage of Click-iT® EdU labeling is that 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 (using acid, heat, or digestion with DNase) to expose the BrdU so that it may be detected with an anti-BrdU antibody. Sample processing for the BrdU assay can result in signal alteration of the cell cycle distribution as well as the destruction of antigen recognition sites when using the acid denaturation method. In contrast, the EdU cell proliferation kit is compatible with cell cycle dyes (Figure 2). The EdU assay can also be multiplexed with antibodies against surface and intracellular markers. However, some reagents or antibody conjugates may not be compatible with the Click-iT® EdU detection reaction and may need some additional steps to ensure compatibility (see Table1).

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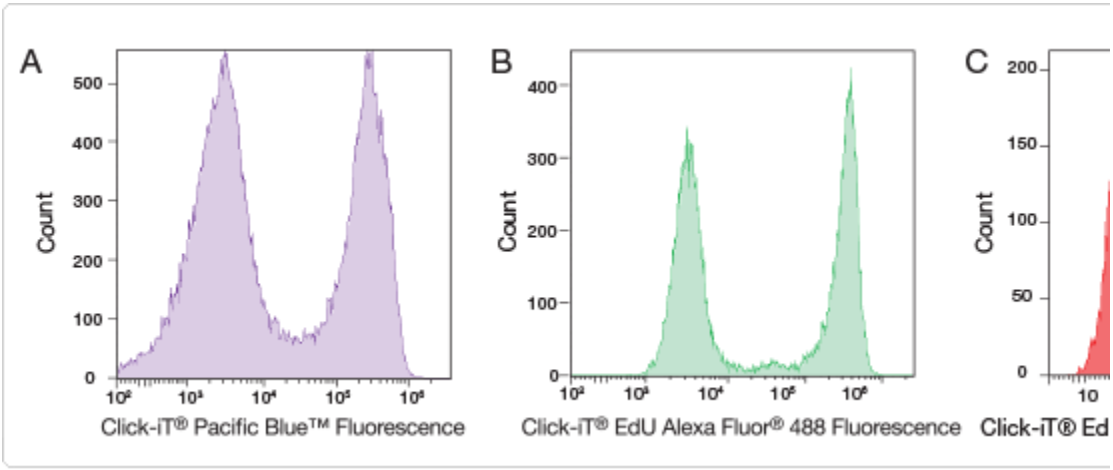

Figure 1. Fluorescence signal from Pacific Blue™, Alexa Fluor® 488, and Alexa Fluor® 647 Click-iT® EdU Flow Cytometry Assay Kits. Jurkat (human T-cell leukemia) cells were treated with 10 µM EdU for 2 hours and detected according to the recommended staining protocol. The figures show a clear separation of proliferating cells which have incorporated EdU and nonproliferating cells which have not. Panel A shows data from cells labeled with Pacific Blue™ azide analyzed on an Attune® Acoustic Focusing Cytometer using 405 nm excitation with a 450/40 nm bandpass emission filter; Panel B shows data from cells labeled with Alexa Fluor® 488 azide analyzed on an Attune® Acoustic Focusing Cytometer using 488 nm excitation and a 530/30 nm bandpass emission filter; Panel C shows data from cells labeled with Alexa Fluor® 647 azide analyzed on a flow cytometer using 633 nm excitation and a 660/20 nm bandpass emission filter.

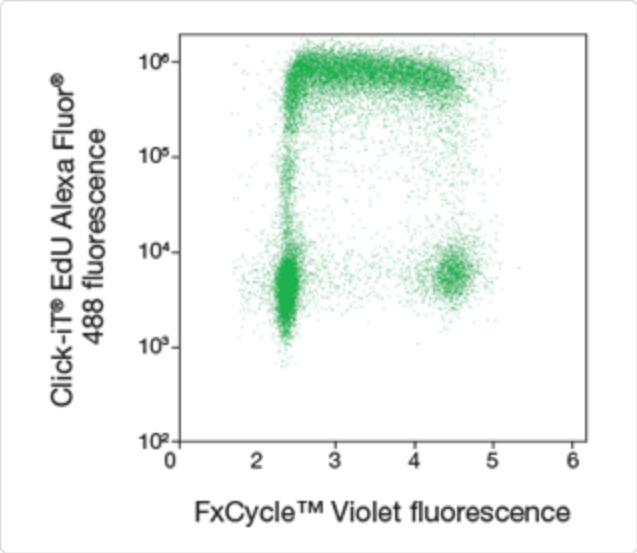

Figure 2. Dual parameter plot of Click-iT® EdU Alexa Fluor® 488 and FxCycle™ Violet. Jurkat (human T-cell leukemia) cells were treated with 10 µM EdU for 2 hours and detected according to the recommended staining protocol. Data was collected and analyzed using an Attune® Acoustic Cytometer using 488 nm excitation and a 530/30 bandpass for detection of the EdU Alexa Fluor® 488 azide and 405 nm excitation and a 450/40 bandpass for detection of the FxCycle™ Violet fluorescence. This figure combines DNA content with EdU; cells that are positive for both labels are in S-phase of the cell cycle.

Table 1. Click-iT® EdU detection reagent compatibility

Fluorescent molecule	**Compatibility***
Qdot® nanocrystals	Use Qdot® nanocrystals **after** the Click-iT® detection reaction.
Fluorescent proteins (GFP)	Use anti-GFP antibodies **before** the Click-iT® detection reaction or use organic dye-based reagents for protein expression detection.
Organic dyes such as Alexa Fluor® dyes, fluorescein (FITC)	Compatible
PerCP, allophycocyanin (APC) and APC-based tandems (i.e., Alexa Fluor® 680-APC)	Compatible
R-phycoerythrin (R-PE) and R-PE based tandems (i.e., Alexa Fluor® 610-RPE)	Use R-PE and R-PE-based tandems **after** the Click-iT® detection reaction.
TC-FlAsH™/TC-ReAsH™ reagents	Detect the tetracysteine (TC) tag with FlAsH™ or ReAsH™ reagents**before** the Click-iT® detection reaction.

*Compatibility indicates whether 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. Not all anti- GFP antibodies recognize the same antigen site. Rabbit and chicken anti-GFP antibodies perform well, but the mouse monoclonal antibodies tested do not generate an acceptable amount of fluorescence and are not recommended for this application.

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Materials

Materials Provided in Kit

Material	Amount	Concentration	Storage*	Stability
EdU (Component A)	10 mg	NA	2–6 ℃ Desiccate Protect from light Do not freeze	When stored as directed, this kit is stable for up to 1 year after receipt.
Alexa Fluor® 488 azide (Cat. no. C10425), Alexa Fluor® 647 azide (Cat. no. C10424), or Pacific Blue™ azide (Cat. no. C10418) (Component B)	1 vial	NA		
Dimethylsulfoxide (DMSO) (Component C)	4.25 mL	NA		
Click-iT® fixative (Component D)	5 mL	4% paraformaldehyde in PBS		
Click-iT® saponin-based permeabilization and wash reagent (Component E)	50 mL	10X solution		
CuSO4 (Component F)	0.5 mL	100 mM aqueous solution		
Click-iT® EdU buffer additive (Component G)	400 mg	NA		
*These storage conditions are appropriate when storing the entire kit upon receipt. For optimal storage conditions for each component, see vial labels. NA = Not applicable.				
Number of assays: Sufficient material is supplied for 50 reactions, based on the protocol below.				
Approximate fluorescence excitation/emission maxima: Pacific Blue™ azide: 410/455 nm; Alexa Fluor® 647 azide: 650/670 nm; Alexa Fluor® 488 azide: 495/519 nm.				

Materials Required but not Provided

- 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS), pH 7.1–pH 7.4
- Buffered saline solution, such as PBS, D-PBS, or TBS
- Deionized water or 18 MΩ purified water
- 12 × 75-mm tubes, or other flow cytometry tubes

Cautions

- 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.
- Click-iT® fixative (Component D) contains paraformaldehyde, which is harmful. Use with appropriate precautions.

- Click-iT® saponin-based permeabilization and wash reagent (Component E) contains sodium azide, which yields highly toxic hydrazoic acid under acidic conditions. Dilute azide compounds in running water before discarding to avoid accumulation of potentially explosive deposits in plumbing.

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Ordering Information

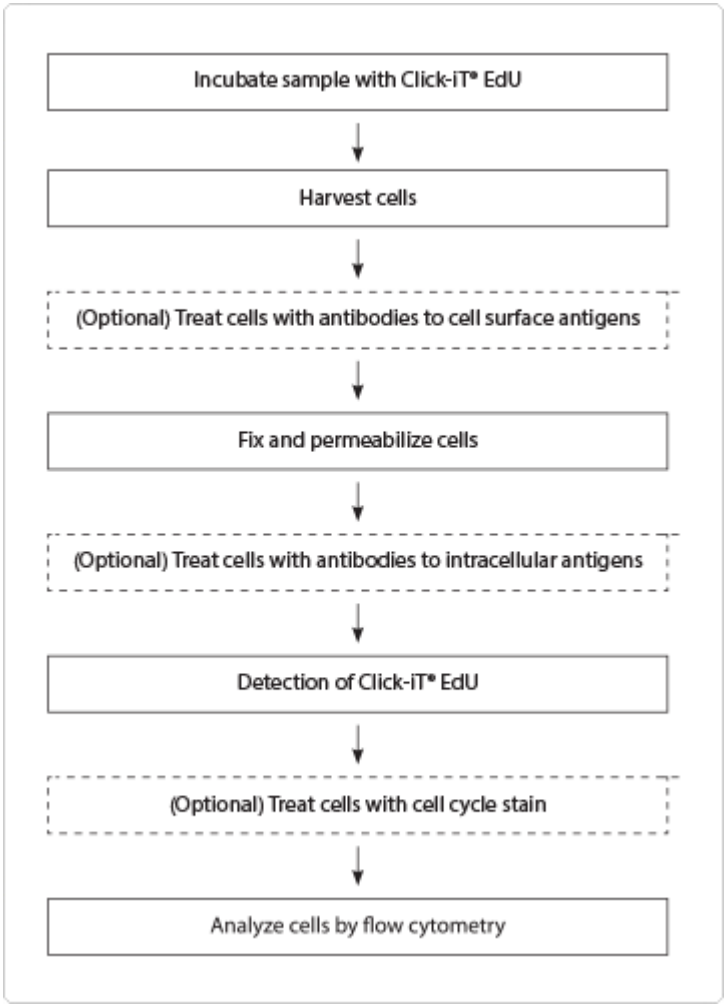
Sku	Product Catalog	Size	Price	Quantity
14190-144	DPBS, no calcium, no magnesium	500 ml	17.80 USD	
14190-250	DPBS, no calcium, no magnesium	10 ×500 ml	155.00 USD	
A10044	EdU (5-ethynyl-2'-deoxyuridine)	50 mg	376.00 USD	
C-10418	Click-iT® EdU Pacific Blue™ Flow Cytometry Assay Kit	50 Assays	500.00 USD	
C-10419	Click-iT® EdU Alexa Fluor® 647 Flow Cytometry Assay Kit	100 Assays	827.00 USD	
C-10420	Click-iT® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit	100 Assays	827.00 USD	
C-10424	Click-iT® EdU Alexa Fluor® 647 Flow Cytometry Assay Kit	50 Assays	500.00 USD	
C-10425	Click-iT® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit	50 Assays	500.00 USD	
F-10347	FxCycle™ Violet Stain	1 kit	323.00 USD	
F-10348	FxCycle™ Far Red Stain	1 kit	317.00 USD	
H3570	Hoechst 33342, Trihydrochloride, Trihydrate - 10 mg⁄mL Solution in Water	10 ml	82.00 USD	
P3566	Propidium Iodide - 1.0 mg⁄mL Solution in Water	10 ml	67.00 USD	
S-10349	SYTOX® AADvanced™ Dead Cell Stain Kit	1 kit	113.00 USD	

V35003	Vybrant® DyeCycle™ Violet Stain	200 µl	359.00 USD	

Add to Cart

Protocol

Summary of Protocol



Preparing Reagents

1.1 Allow vials to warm to room temperature before opening.

1.2 To prepare a 10 mM solution of EdU, add 4 mL of DMSO (Component C) or aqueous solution (PBS) to Component A and mix well. After use, store any remaining stock solution at ≤–20 ℃. When stored as directed, the stock solution is stable for up to 1 year.

1.3 To prepare a working solution of Pacific Blue™ azide (Cat. no. C10418), Alexa Fluor® 647 azide (Cat. no. C10424), or Alexa Fluor® 488 azide (Cat. no. C10425), add 130 µL of DMSO to Component B and mix well.

After use, store any remaining working solution at ≤–20 ℃. When stored as directed, this working solution is stable for up to 1 year.

1.4 To prepare 500 mL of 1X Click-iT® saponin-based permeabilization and wash reagent, add 50 mL of Component E to 450 mL of 1% BSA in PBS. Smaller amounts can be prepared by diluting a volume of Component E 1:10 with 1% BSA in PBS. After use, store any remaining solutions at 2–6˚C. When stored as directed, the 1X solution is stable for 6 months and the 10X solution is stable for 12 months after receipt. Note: Component E contains sodium azide (see Cautions).

1.5 To make a 10X stock solution of the Click-iT® EdU buffer additive (Component G), add 2 mL of deionized water to the vial and mix until the Click-iT® EdU buffer additive is fully dissolved. After use, store any remaining stock solution at ≤–20˚C. When stored as directed, the stock solution is stable for up to 1 year.

Labeling Cells with EdU

The following protocol was developed with Jurkat cells, a human T cell line, and using an EdU concentration of 10 μM, and can be adapted for any 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. If currently using a BrdU based assay for cell proliferation, a similar concentration to BrdU is a good starting concentration for EdU. If using whole blood as the sample, we recommend heparin as the anticoagulant for collection.

2.1 Suspend the cells in an appropriate tissue culture medium to obtain optimal conditions for cell growth. Disturbing the cells by temperature changes or washing prior to incubation with EdU slows the growth of the cells during incorporation.

2.2 Add EdU to the culture medium at the desired final concentration and mix well. We recommend a starting concentration of 10 μM for 1–2 hours. For longer incubations, use lower concentrations. For shorter incubations, higher concentrations may be required. For a negative staining control, include cells from the same population that have not been treated with EdU.

2.3 Incubate under conditions optimal for cell type for the desired length of time. Altering the amount of time the cells are exposed to EdU or subjecting the cells to pulse labeling with EdU allows the evaluation of various DNA synthesis and proliferation parameters. Effective time intervals for pulse labeling and the length of each pulse depend on the cell growth rate.

2.4 Harvest cells and proceed immediately to step 3.1 if performing antibody surface labeling; otherwise continue to step 4.1.

Staining Cell-Surface Antigens with Antibodies (Optional)

3.1 Wash cells once with 3 mL of 1% BSA in PBS, pellet cells by centrifugation, and remove supernatant.

3.2 Dislodge the pellet and resuspend cells at 1 × 107 cells/mL in 1% BSA in PBS.

3.3 Add 100 μL of cell suspension or whole blood sample to flow tubes.

3.4 Add surface antibodies and mix well (Table 2, page 3). Note: Do not use PE, PE-tandem, or Qdot® antibody conjugates before performing the click reaction; wait until step 6.1 for labeling with these fluorophores.

3.5 Incubate for the recommended time and temperature, protected from light. 3.6 Proceed to step 4.1 for cell fixation.

Fixation and Permeabilization

The Click-iT® saponin-based permeabilization and wash reagent can be used with whole blood or cell suspensions containing red blood cells, as well as with cell suspensions containing more than one cell type. This permeabilization and wash reagent maintains the morphological light scatter characteristics of leukocytes while lysing red blood cells.

4.1 Wash the cells once with 3 mL of 1% BSA in PBS, pellet the cells, and remove the supernatant.

4.2 Dislodge the pellet, add 100 µL of Click-iT® fixative (Component D), and mix well.

4.3 Incubate the cells for 15 minutes at room temperature, protected from light.

4.4 Wash the cells with 3 mL of 1% BSA in PBS, pellet the cells, and remove the supernatant. Repeat the wash step if red blood cells or hemoglobin are present in the sample. Remove all residual red blood cell debris and hemoglobin before proceeding.

4.5 Dislodge the cell pellet and resuspend the cells in 100 µL of 1X Click-iT® saponin-based permeabilization and wash reagent (prepared in step 1.4), and mix well. Incubate the cells for 15 minutes or proceed directly to step 5.1 for click labeling.

Click-iT® Reaction

5.1 Prepare 1X Click-iT® EdU buffer additive by diluting the 10X stock solution (prepared in step 1.5) 1:10 in deionized water.

5.2 Prepare the Click-iT® reaction cocktail according to Table 3.

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

Table 2. Click-iT® EdU Reaction Cocktails

	Number of Reactions						
Reaction Components	**1**	**2**	**5**	**10**	**15**	**30**	**50**
PBS, D-PBS, or TBS	438 µL	875 µL	2.19 mL	4.38 mL	6.57 mL	13.2 mL	21.9 mL
CuSO4 (Component F)	10 µL	20 µL	50 µL	100 µL	150 µL	300 µL	500 µL
Fluorescent dye azide (prepared in step 1.3)	2.5 µL	5 µL	12.5 µL	25 µL	37.5 µL	75 µL	125 µL

Reaction Buffer Additive (prepared in step 5.1)	50 µL	100 µL	250 µL	500 µL	750 µL	1.5 mL	2.5 mL
Total reaction volume	500 µL	1 mL	2.5 mL	5 mL	7.5 mL	15 mL	25 mL

5.3 Add 0.5 mL of Click-iT® reaction cocktail to each tube and mix well.

5.4 Incubate the reaction mixture for 30 minutes at room temperature, protected from light.

5.5 Wash the cells once with 3 mL of 1X Click-iT® saponin-based permeabilization and wash reagent (prepared in step 1.4), pellet the cells, and remove the supernatant. Dislodge the cell pellet and resuspend the cells in 100 µL of 1X Click-iT® saponin-based permeabilization and wash reagent, if proceeding with intracellular antibody labeleing in step 6.1. Otherwise, add 500 µL of 1X Click-iT® saponin-based permeabilization and wash reagent and proceed with step 7.1 for staining the cells for DNA content, or with step 8.1 for analyzing the cells on a flow cytometer.

Staining Cells for DNA Content (Optional)

7.1 If necessary, add Ribonuclease A to each tube and mix (Table 3).

Table 3. Click-iT® EdU compatibility with DNA content stains

	Click-iT® EdU Stain Compatibility			
DNA Content Stain	**Pacific Blue™**	**Alexa Fluor® 647**	**Alexa Fluor® 488**	**RNase required?**
FxCycle™ Violet	No	Yes	Yes	No
Propidium iodide (PI)	Yes	Yes	No*	Yes
SYTOX® AADvanced™	Yes	Yes	Yes	Yes
FxCycle™ Far Red	Yes	No	Yes	Yes

7.2 Add the appropriate DNA stain to each tube, mix well, and incubate as recommended for each DNA stain.

Analysis by Flow Cytometry

If measuring total DNA content on a traditional flow cytometer using hydrodynamic focusing, use a low flow rate during acquisition. If using the Attune® Acoustic Focusing Cytometer, all collection rates may be used without loss of signal integrity if the event rate is kept below 10,000 events per second. However, for each sample within an experiment, the same collection rate and cell concentration should be used. The fluorescent signal generated by DNA content stains is best detected with linear amplification. The fluorescent signal generated by Click-iT® EdU labeling is best detected with logarithmic amplification.

8.1 Analyze the cells using a flow cytometer.

- For the detection of EdU with Pacific Blue™ azide, use 405 nm excitation with a violet emission filter (450/50 nm or similar).

- For the detection of EdU with Alexa Fluor® 647 azide use 633/635 nm excitation with a red emission filter (660/20 nm or similar).
- For the detection of EdU with Alexa Fluor® 488 azide, use 488 nm excitation with a green emission filter (530/30 nm or similar).

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References

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