invitrogen

ThermoFisher SCIENTIFIC

Click-iT™ Plus EdU Flow Cytometry Assay Kits, 50 tests

Catalog nos. C10632, C10634, C10636, C10645, C10646

Table 1. Contents and storage

Material	Amount	Concentration	Storage*	Stability
EdU (Component A)	10 mg	NA	• 2–8°C • Desiccate • Protect from light • DO NOT FREEZE	When stored as directed the product is stable for up to 1 year after receipt.
Alexa Fluor™ 350 picolyl azide (Cat. no. C10645), Alexa Fluor™ 488 picolyl azide (Cat. no. C10632), Alexa Fluor™ 594 picolyl azide (Cat. no. C10646), Alexa Fluor™ 647 picolyl azide (Cat. no. C10634), or Pacific Blue™ picolyl azide (Cat. no. C10636) (Component B)	130 µL	DMSO solution		
Dimethylsulfoxide (DMSO) (Component C)	4.5 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		
Copper protectant (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 assays based on the protocol below.

Approximate fluorescence excitation and emission maxima: Alexa Fluor™ 350 picolyl azide: 350/440 nm; Alexa Fluor™ 488 picolyl azide: 495/519 nm; Alexa Fluor™ 594 picolyl azide: 532 or 561/617 nm; Alexa Fluor™ 647 picolyl azide: 650/670 nm; Pacific Blue™ picolyl azide: 404/450 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 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™ Plus 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 a picolyl azide and an alkyne. In this application, the alkyne is found in the ethynyl moiety of EdU, while the picolyl azide is coupled to Alexa Fluor™ 350 dye, Alexa Fluor™ 488 dye, Alexa Fluor™ 594 dye, Alexa Fluor™ 647 dye, or Pacific Blue™ dye. Standard flow cytometry methods are used for determining the percentage of S-phase cells in the population (Figures 1–3).

The advantage of Click-iT™ Plus EdU labeling is that the small size of the picolyl 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™ Plus 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 Click-iT™ Plus EdU cell proliferation kit is compatible with cell cycle dyes, R-PE, R-PE tandems, and fluorescent proteins such as GFP, RFP, and mCherry. Figure 1 (below) shows Click-iT™ Plus EdU Alexa Fluor™ 488 multiplexed with R-PE, Figure 2 (page 3) demonstrates Click-iT™ Plus EdU Alexa Fluor™ 350 multiplexed with Pacific Orange™ and FxCycle™ Far Red, and Figure 3 (page 3) shows Click-iT™ Plus EdU Alexa Fluor™ 647 multiplexed with GFP and FxCycle™ Violet. The EdU assay can also be multiplexed with antibodies against surface and intracellular markers (Table 2, page 4).

Figure 1. Fluorescence signal from Alexa Fluor™ 488 Click-iT™ Plus EdU Flow Cytometry Assay Kits and CD3 mouse anti-Human mAb PE conjugate. Jurkat (human T-cell leukemia) cells were treated with 10 µM EdU for 2 hours, stained with CD3 mouse anti-Human mAb PE conjugate (Cat. no. MHCD0304) 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 Alexa Fluor™ 488 picolyl azide analyzed on an Attune™ Acoustic Focusing Cytometer using 488 nm excitation and a 530/30 nm bandpass emission filter; **Panel B** shows the same cells using 635 nm excitation and a 574/26 nm bandpass emission filter. The black outlined histogram is the cells stained with CD3 mouse anti-Human mAb PE conjugate and Click-iT™ Plus EdU Alexa Fluor™ 488 picolyl azide. The gray outlined histogram is the CD3 mouse anti-Human mAb PE conjugate positive control cells treated the same but without copper in the reaction.

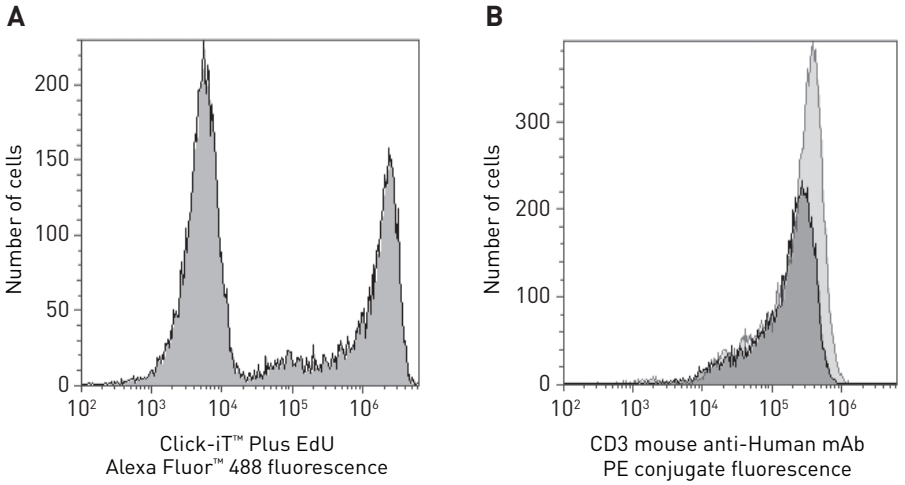


Figure 2. Fluorescence signal and dual parameter plots from Alexa Fluor™ 350 Click-iT™ Plus EdU Flow Cytometry Assay Kit, CD45-Pacific Orange™ and FxCycle™ Far Red. Jurkat (human T-cell leukemia) cells were treated with 10 µM EdU for 2 hours, stained with CD45-Pacific Orange™ (Cat. no. MHCD4530), and detected according to the recommended staining protocol. The figures show a clear separation of proliferating cells which have incorporated EdU and non-proliferating cells which have not. **Panel A** shows data from cells labeled with Alexa Fluor™ 350 picolyl azide analyzed on a BD™ LSRII flow cytometer using UV excitation and a 450/50 nm bandpass emission filter; **Panel B** shows the same cells using 488 nm excitation and a 530/30 nm bandpass emission filter for detection of the CD45-Pacific Orange™ and UV excitation, and a 450/50 nm bandpass emission filter for detection of the Alexa Fluor™ 350 picolyl azide; **Panel C** shows the dual parameter plot of the Click-iT™ Plus EdU Alexa Fluor™ 350 and FxCycle™ Far Red. Data were collected and analyzed using a BD™ LSRII flow cytometer using UV excitation and a 450/50 nm bandpass emission filter for detection of the Alexa Fluor™ 350 picolyl azide and 633 nm excitation and a 660/20 nm bandpass emission filter for detection of the FxCycle™ Far Red fluorescence. This figure combines DNA content with EdU; cells that are positive for both labels are in S-phase of the cell cycle.

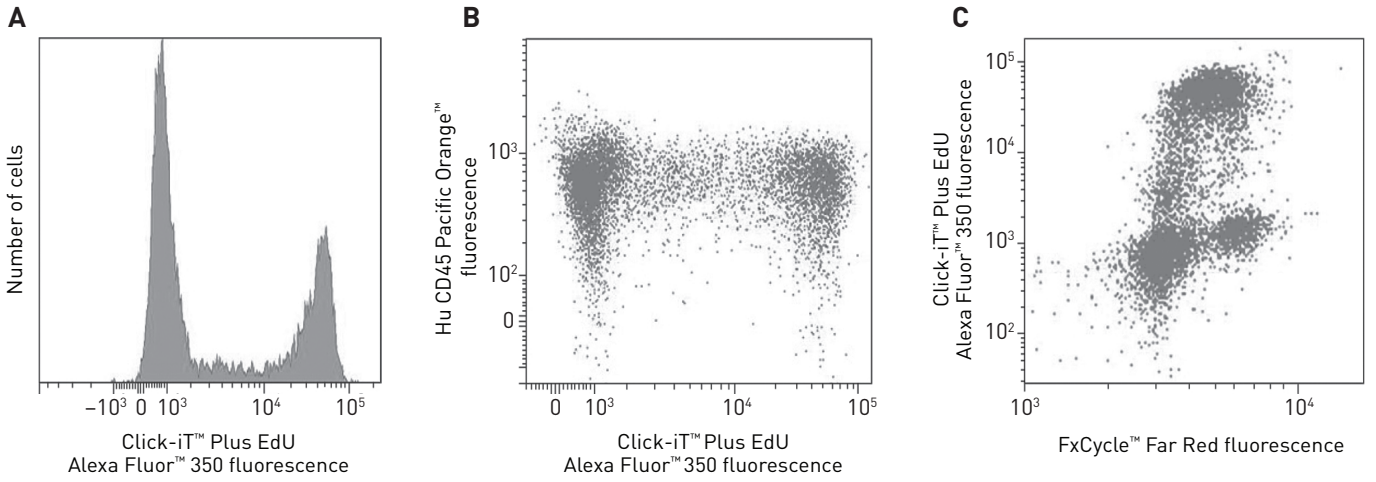


Figure 3. Fluorescence signal and dual parameter plot of Alexa Fluor™ 647 Click-iT™ Plus EdU Flow Cytometry Assay Kits, Green Fluorescent Protein, and FxCycle™ Violet. A375 (human malignant melanoma) cells were treated with 10 µM EdU for 2 hours and detected according to the recommended staining protocol. 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 Alexa Fluor™ 647 picolyl azide analyzed on an Attune™ Acoustic Focusing Cytometer using 635 nm excitation and a 660/20 nm bandpass emission filter; **Panel B** shows the same cells using 488 nm excitation and a 530/30 nm bandpass emission filter. The black outlined histogram is the GFP-expressing cells and Click-iT™ Plus EdU Alexa Fluor™ 647 picolyl azide. The gray outlined histogram is the GFP-expressing positive control cells treated the same but without copper in the reaction. **Panel C** shows the dual parameter plot of the Click-iT™ Plus EdU Alexa Fluor™ 647 and FxCycle™ Violet. Data were collected and analyzed using an Attune™ Acoustic Cytometer using 635 nm excitation and a 660/20 nm bandpass emission filter for detection of the EdU Alexa Fluor™ 647 picolyl azide and 405 nm excitation and a 450/40 bandpass emission filter 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.

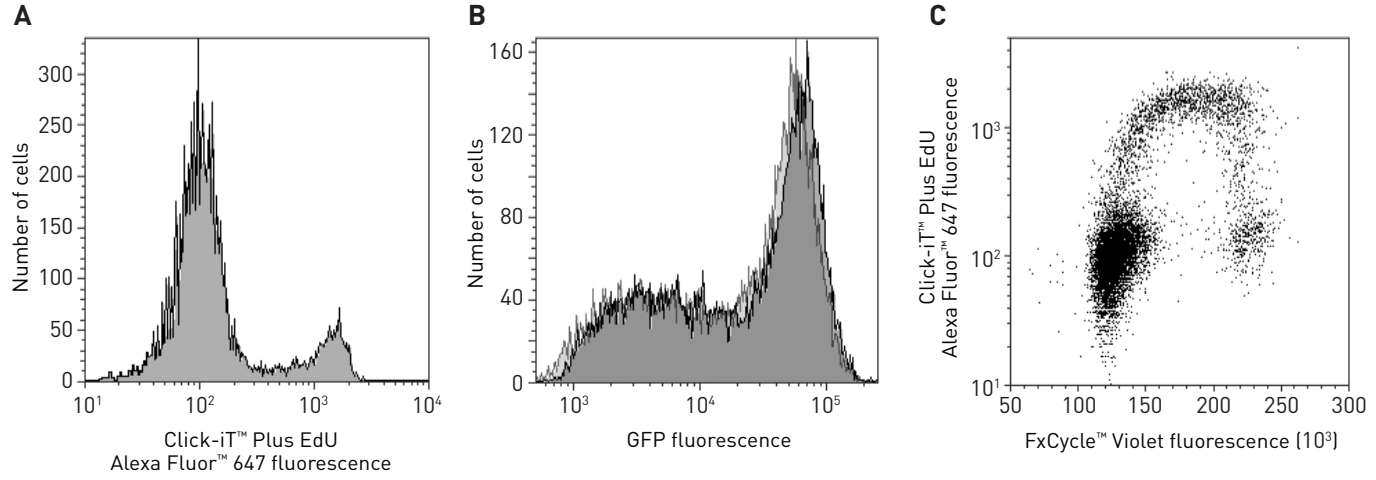


Table 2. Click-iT™ Plus EdU detection reagent compatibility.

Fluorescent molecule	Compatibility*
R-phycoerythrin (R-PE) and R-PE based tandems (i.e., Alexa Fluor™ 610-RPE)	Compatible, except Alexa Fluor™ 594 with R-PE-Cy®7 tandems
Fluorescent proteins (GFP)	Compatible
PerCP, allophycocyanin (APC) and APC-based tandems (i.e. Alexa Fluor™ 680-APC)	Compatible
Organic dyes such as Alexa Fluor™ dyes, fluorescein (FITC)	Compatible
Qdot™ nanocrystals	Use Qdot™ nanocrystals **after** the Click-iT™ Plus detection reaction.
*Compatibility indicates whether the fluorescent molecule itself or the detection methods involve components that are unstable in the presence of the copper catalyst used for the Click-iT™ Plus EdU detection reaction.	

Before you begin

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 (in Components B and 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.

Prepare 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°C. When stored as directed, the stock solution is stable for up to 1 year.

1.3 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 2–8°C. When stored as directed, the 1X solution is stable for 6 months and the 10X solution is stable for 12 months after receipt.

Important! Component E contains sodium azide (see Cautions, above).

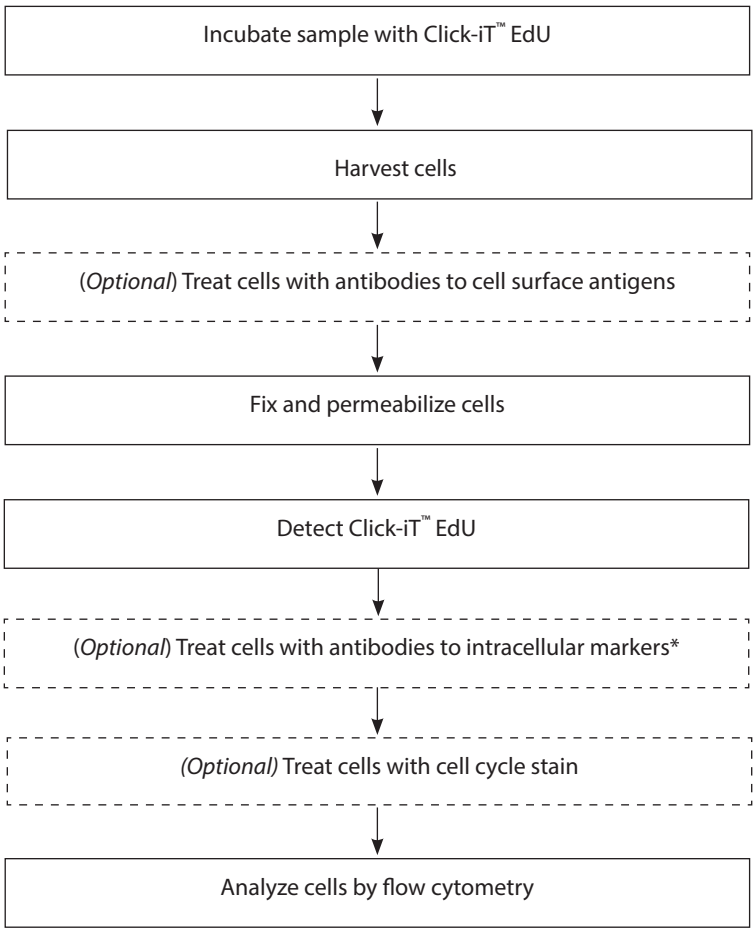
Note: There is a degree of variability that has been observed in the color of the saponin solution (component E), from light yellow to dark brown. There is no difference in the kit performance by flow cytometry for the different colors of saponin that are observed.

1.4 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.

Experimental protocols

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.

Figure 4. Workflow diagram for the Click-iT™ Plus EdU Flow Cytometry Assay Kits



* You may treat cells with antibodies to intracellular markers *before* detecting Click-iT™ EdU. See the following procedures for detailed instructions.

Label cells with EdU

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.

Stain 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 1×10^7 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 4).

Note: Do not use 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.

Fix and permeabilize

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 1.3), and mix well. Incubate the cells for 15 minutes or proceed directly to step 5.1 for click labeling or to step 6.1 for treating cells with antibodies to intracellular antigens (steps 6.1–6.3) followed by click labeling.

Click-iT™ reaction

5.1 Prepare 1X Click-iT™ EdU buffer additive by diluting the 10X stock solution (prepared in step 1.4) 1:10 in deionized water.

5.2 Prepare the Click-iT™ Plus reaction cocktail according to Table 3 below.

Table 3. Click-iT™ Plus reaction cocktails

Reaction components	Number of reactions						
	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
Copper protectant (Component F)	10 µL	20 µL	50 µL	100 µL	150 µL	300 µL	500 µL
Fluorescent dye picolyl azide	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

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

5.3 Add 0.5 mL of Click-iT™ Plus reaction cocktail to each tube (from step 4.5) and mix well.

Note: The total volume for each reaction mixture is 600 µL.

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 1.3), 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 labeling 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.

Stain intracellular or surface antigens (optional)

6.1 Add antibodies against intracellular antigens or against surface antigens that use Qdot™ antibody conjugates. Mix well.

6.2 Incubate the tubes for the time and temperature required for antibody staining, protected from light.

6.3 Wash the cells according to the following conditions, depending on whether you have already detected Click-iT™ EdU:

- If you have detected Click-iT™ EdU (i.e. you have already followed **Click-iT™ reaction** steps 5.1–5.5), wash each tube with 3 mL of 1X Click-iT™ saponin-based permeabilization and wash reagent (prepared in step 1.3), pellet the cells, and remove the supernatant. Dislodge the cell pellet and resuspend the cells in 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.

or

- If you have not yet detected Click-iT™ EdU, wash the cells once with 3 mL of 1% BSA in PBS, pellet the cells, and remove the supernatant. Dislodge the cell pellet and add 100 uL of 1x Click-iT™ saponin-based permeabilization and wash reagent (prepared in step 1.3) and mix well before proceeding to step 5.1 for click labeling.

Stain cells for DNA content (optional)

7.1 If necessary, add Ribonuclease A to each tube and mix (Table 4, below).

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

Table 4. Click-iT™ Plus EdU compatibility with DNA content stains

DNA content stain	Click-iT™ Plus EdU stain compatibility					RNase required?
	Alexa Fluor™ 350 picolyl azide	Alexa Fluor™ 488 picolyl azide	Alexa Fluor™ 594 picolyl azide	Alexa Fluor™ 647 picolyl azide	Pacific Blue™ picolyl azide	
FxCycle™ PI/RNase	Yes	Yes	Yes	Yes	Yes	No
FxCycle™ Violet	No	Yes	Yes	Yes	No	No
FxCycle™ Far Red	Yes	Yes	Yes	No	Yes	Yes
SYTOX™ AADvanced™	Yes	Yes	Yes	Yes	Yes	Yes
Propidium iodide (PI)	Yes	No	Yes	Yes	Yes	Yes

Analyze 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™ or Attune™ NxT Flow 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™ Plus EdU labeling is best detected with logarithmic amplification.

8.1 Analyze the cells using a flow cytometer.

- For the detection of EdU with Alexa Fluor™ 350 picolyl azide, use 350 nm excitation with a 440 emission filter (450/50 nm or similar).
- For the detection of EdU with Alexa Fluor™ 488 picolyl azide, use 488 nm excitation with a green emission filter (530/30 nm or similar).
- For the detection of EdU with Alexa Fluor™ 594 picolyl azide, use 532 or 561 nm excitation with a 620 emission filter (620/15 nm or similar).
- For the detection of EdU with Alexa Fluor™ 647 picolyl azide, use 633/635 nm excitation with a red emission filter (660/20 nm or similar).
- For the detection of EdU with Pacific Blue™ picolyl azide, use 405 nm excitation with a violet emission filter (450/40 nm or similar).

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.** BioTechniques 44, 927 (2008); **6.** Curr Protoc Cytom 55, 7.38.1 (2011).

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

Cat. no.	Product name	Unit size
C10645	Click-iT™ Plus EdU Alexa Fluor™ 350 Flow Cytometry Assay Kit *50 assays*	1 kit
C10632	Click-iT™ Plus EdU Alexa Fluor™ 488 Flow Cytometry Assay Kit *50 assays*	1 kit
C10646	Click-iT™ Plus EdU Alexa Fluor™ 594 Flow Cytometry Assay Kit *50 assays*	1 kit
C10634	Click-iT™ Plus EdU Alexa Fluor™ 647 Flow Cytometry Assay Kit *50 assays*	1 kit
C10636	Click-iT™ Plus EdU Pacific Blue™ Flow Cytometry Assay Kit *50 assays*	1 kit
Related products		
C10635	Click-iT™ Plus EdU Alexa Fluor™ 647 Flow Cytometry Assay Kit *100 assays*	1 kit
C10633	Click-iT™ Plus EdU Alexa Fluor™ 488 Flow Cytometry Assay Kit *100 assays*	1 kit
A10044	EdU (5-ethynyl-2´-deoxyuridine)	50 mg
B35129	BrdU mouse monoclonal antibody (Clone MoBU-1) - Pacific Blue™ *for flow cytometry* *100 tests*	1 each
B35139	BrdU mouse monoclonal antibody (Clone MoBU-1) Alexa Fluor™ 488 *for flow cytometry* *100 tests*	1 each
B35140	BrdU mouse monoclonal antibody (Clone MoBU-1) Alexa Fluor™ 647 *for flow cytometry* *100 tests*	1 each
B35141	BrdU mouse monoclonal antibody (Clone MoBU-1) unconjugated *for flow cytometry* *100 tests*	1 each
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
C10354	Click-iT™ EdU Alexa Fluor™ 594 HCS Assay *2-plate size*	1 kit
C10356	Click-iT™ EdU Alexa Fluor™ 647 HCS Assay *2-plate size*	1 kit
F10347	FxCycle™ Violet Stain *for flow cytometry* *500 assays*	1 kit
F10348	FxCycle™ Far Red Stain *for flow cytometry* *500 assays*	1 kit
F10797	FxCycle™ PI/RNase Staining Solution *200 assays*	100 mL
H3570	Hoechst™ 33342, trihydrochloride, trihydrate *10 mg/mL solution in water*	10 mL
P3566	Propidium Iodide – 1.0 mg/mL solution in water	10 mL
S10349	SYTOX™ AADvanced™ dead cell stain *for 488 excitation* *for flow cytometry* *100 tests*	1 kit
V35003	Vybrant™ DyeCycle™ Violet stain *5 mM in water* *200 assays*	200 µL
12091-021	RNase A (20 mg/mL)	10 mL
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 x 500 mL

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These high-quality reagents and materials must be used by, or directly under the supervision of, a technically qualified individual experienced in handling potentially hazardous chemicals. Read the Safety Data Sheet provided for each product; other regulatory considerations may apply.

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