Materials Provided in Kit

Material	Amount	Concentration	Storage*	Stability
EdU *(Component A)*	10 mg	NA	2–6°C 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.

Summary of Protocol

- Incubate sample with Click-iT® EdU
- Harvest cells
- *(Optinal) Treat cells whith antibodies to cell surface antigens*
- Fix and permeabilize cells
- *(Optinal) Treat cells whith antibodies to intracellular antigens*
- Detection of Click-iT® EdU
- *(Optinal) Treat cells whith cell cycle stain*
- Analyze cells by flow cytometry

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°C.
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°C.
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 4).*
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 2*.

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

Table 2. Click-iT® EdU 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
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 labeling.
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

Use a low flow rate during acquisition, 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).