Click-iT™ EdU Flow Cytometry Assay Kits

Table 1. Contents and storage information.

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.
Alexa Fluor® 488 azide (Cat. no. C35002), Alexa Fluor® 647 azide (Cat. no. A10202), or Pacific Blue™ azide (Cat. no. A10034) (Component B)	1 vial	NA		
Dimethylsulfoxide (DMSO, Component C)	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		
Click-iT™ Triton® X-100-based permeabilization reagent (Component F)	5 mL	1X solution in PBS		
Click-iT™ EdU reaction buffer (Component G)	4 mL	10X solution containing Tris-buffered saline		
$CuSO_4$ (Component H)	550 µL	100 mM aqueous solution		
Click-iT™ EdU buffer additive (Component I)	800 mg	NA		
Click-iT™ EdU CellCycle 633-red or CellCycle 405-blue (Component J)	1 vial	NA		
Click-iT™ EdU CellCycle 488-red (7-AAD) (Component K, Cat. no. A10034, C35002)	1 vial	NA		
Propidium iodide (PI) (Component K, Cat. no. A10202)	1 vial	1 mg/mL solution in water		
Ribonuclease A (MW ~13,700, Component L)	260 µL	20 mg/mL		

*These storage conditions are appropriate when storing the entire kit upon receipt. For optimal storage conditions for each component, see labels on the vials. †Contains 0.09% sodium azide. NA = Not applicable.

Number of assays: Sufficient material is supplied for 50 assays, based on the protocol below.

Approximate fluorescence excitation/emission maxima: Alexa Fluor® 488 azide: 495/519 nm; Alexa Fluor® 647 azide: 650/670 nm; Pacific Blue™ azide: 410/455 nm; Click-iT™ EdU CellCycle 633-red: 640/658 nm, bound to DNA; Click-iT™ EdU CellCycle 488-red: 546/647 nm, bound to DNA; Click-iT™ EdU CellCycle 405-blue: 369/437 nm, bound to DNA; Propidium iodide: 535/617 nm, bound to DNA

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 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™ 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 EdU contains the alkyne, while the Alexa Fluor® 488 dye, Alexa Fluor® 647 dye, or Pacific Blue™ dye contains the azide. Standard flow cytometry methods are used for determining the percentage of cells in the population that are in S-phase (Figures 1 and 4).

The advantages of Click-iT™ EdU labeling are readily evident while performing the assay (Figures 2 and 3). 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 HCl, 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 from the cell cycle distribution as well as destruction of antigen recognition sites when using the HCl method. In contrast, the easy to use EdU cell proliferation kit is compatible with cell cycle dyes. This EdU assay kit can also be multiplexed with antibodies against surface and intracellular markers. However, some of these reagents or antibodies may not be compatible with the Click-iT™ EdU detection reagent and may need some additional steps to make the reagent or antibody compatible with the Click-iT™ EdU detection reagent (see Table 2 for details).

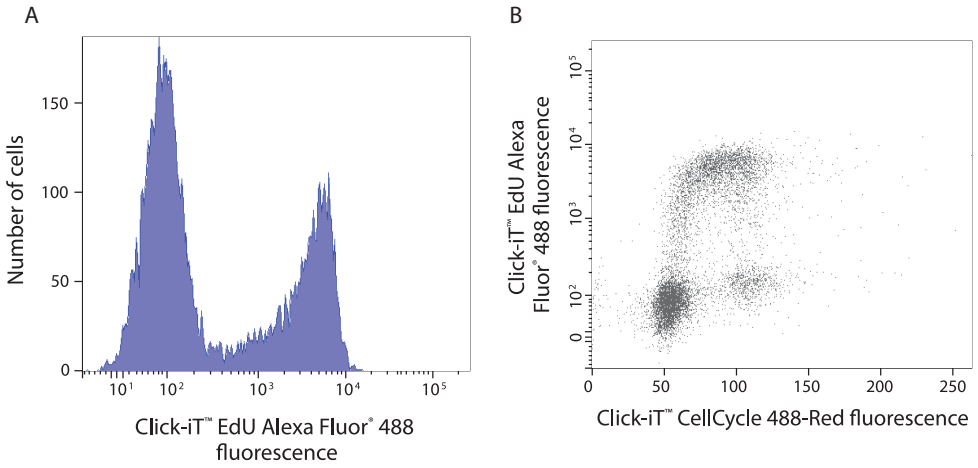

Figure 1. Dual parameter plot of Jurkat cells with Alexa Fluor® 488 azide and Click-iT™ CellCycle 488-red. Jurkat cells were treated with 10 µM EdU for one hour. Panel A shows cells labeled with Alexa Fluor® 488 azide using 488 nm excitation with a 530/30 nm bandpass; clear separation of proliferating cells which have incorporated EdU and nonproliferating cells is demonstrated. Panel B combines DNA content with EdU cells that are co-positive for both labels providing the percentage of cells in S-phase of DNA synthesis.

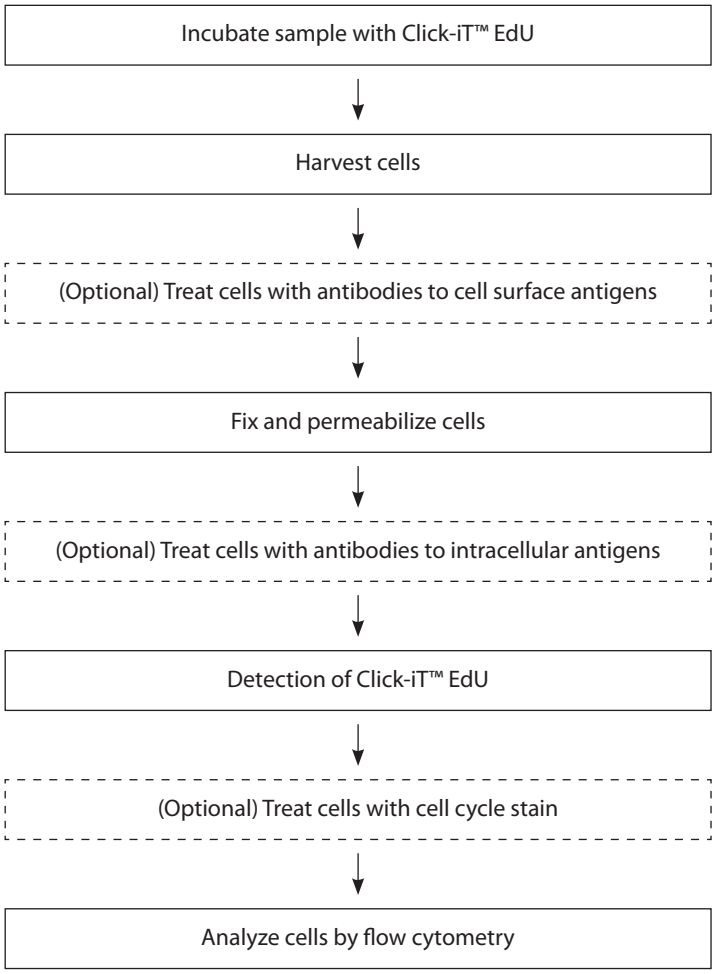

Figure 2. Workflow diagram for the Click-iT™ EdU Flow Cytometry Assay..

The kits contain all of the components needed to label and detect the incorporated EdU, as well as to perform cell cycle analysis on samples from whole blood, adherent cells, or suspension cells (Figures 1 and 4). For cell cycle analysis, each kit is supplied with two different cell cycle dyes compatible with the fluorescence excitation and emission of the EdU detection reagent. Sufficient reagents are provided for performing 50 flow cytometry-based assays using 0.5 mL reaction buffer per assay.

Before You Begin

Materials Required but Not Provided

- 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) (1% BSA in PBS), pH 7.1–pH 7.4
- Deionized water or 18 megohm purified water
- Flow tubes

Table 2. 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, whereas the mouse monoclonal antibodies tested do not generate an acceptable amount of fluorescence, and are not recommended for this application.

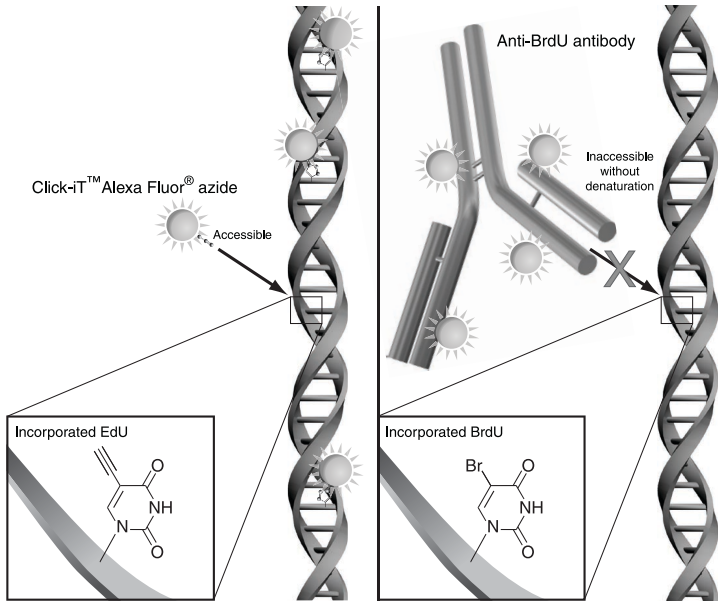

Figure 3. Detection of the incorporated EdU with the Alexa Fluor® azide versus incorporated BrdU with an anti-BrdU antibody. The small size of the Alexa Fluor® azide eliminates the need to denature the DNA for the EdU detection reagent to gain access to the nucleotide.

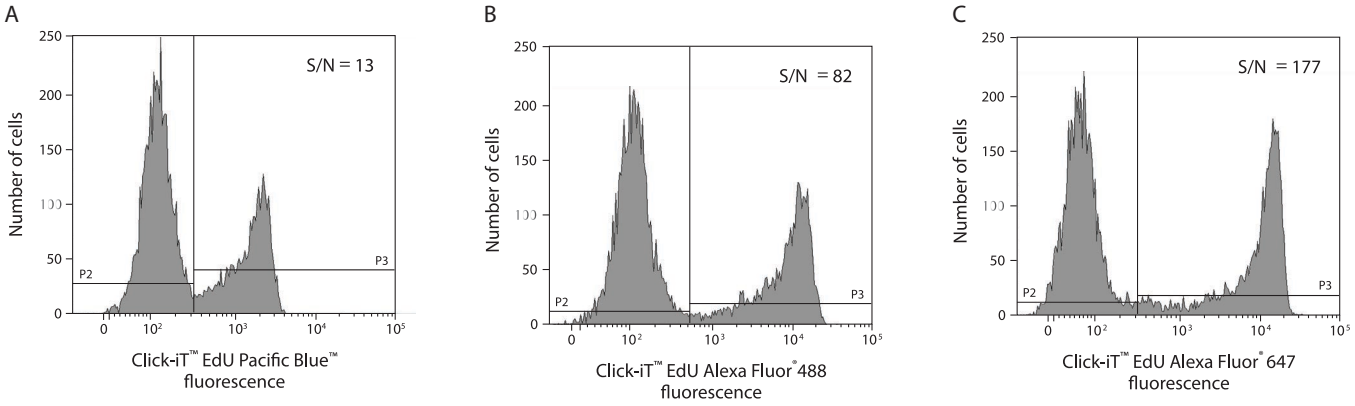

Figure 4. Comparison of the fluorescence signal from Alexa Fluor® 488, Alexa Fluor® 647, and Pacific Blue™ Click-iT™ EdU flow cytometry assay kits. Jurkat (human T-cell leukemia) cells were treated with 10 μM EdU for 2 hours and detected according to the staining protocol. Panel A shows data from cells labeled with Pacific Blue™ azide using 405 nm excitation with a 450/50 nm bandpass; Panel B shows data from cells labeled with Alexa Fluor® 488 azide using 488 nm excitation and a 530/30 nm bandpass emission filter; Panel C shows data from cells labeled with Alexa Fluor® 647 azide using 633 nm excitation and a 660/20 nm bandpass. Signal to background (S/N) was calculated by dividing the median Click-iT™ EdU positive population (P3) by the median non-proliferating population (P2) as indicated by the markers.

Cautions

Use the Click-iT™ EdU CellCycle 633-red or CellCycle 405-blue (Component J) and Click-iT™ EdU CellCycle 488-red (7-AAD) or propidium iodide (Component K) with appropriate precautions as these compounds are known or suspected mutagens.

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

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™ saponin-based permeabilization and wash reagent (Component E) contains 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.

Preparing the Stock Solutions

Not all of the following stock or working solutions prepared are used in every experiment. This kit includes two dyes to measure DNA content and cell cycle distribution. If cell cycle analysis is of interest, use only one of the dyes that is appropriate for the available excitation lasers. This kit also contains two different permeabilization buffers; use only the buffer that is appropriate for the sample type and experiment.

1.1 Allow vials to warm to room temperature before opening.

1.2 To make a 10 mM solution of EdU (Component A), add 4 mL DMSO (Component C) or aqueous solution (*i.e.*, buffer, saline) 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 make a working solution of Alexa Fluor® or Pacific Blue™ azide:

- Add 260 μL DMSO (Component C) to Alexa Fluor® 488 azide, (Component B, Cat. no C35002) and mix well.

OR

- Add 156 µL DMSO (Component C) to Alexa Fluor® 647 azide (Cat. no. A10202) or Pacific Blue™ azide (Component B, Cat. no. A10034) 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 make 500 mL 1X Click-iT™ saponin-based permeabilization and wash reagent (Component E), add 50 mL Component E to 450 mL 1% BSA in PBS. To make smaller amounts of Component E, dilute volumes from the Component E bottle 1:10 with 1% BSA in PBS. After use, store any remaining 1X solution at 2–6˚C. When stored as directed, the 1X solution is stable for 6 months.

Note: This solution contains sodium azide.

1.5 To make 40 mL 1X Click-iT™ EdU reaction buffer (Component G), transfer all of the solution (4 mL) in the Component G bottle to 36 mL of deionized water. Rinse the Component G bottle with some of the diluted Click-iT™ EdU reaction buffer to allow transfer of all of the 10X concentrate. To make smaller amounts of 1X Click-iT™ EdU reaction buffer, dilute volumes from the Component G bottle 1:10 with deionized water. After use, store any remaining 1X solution at 2–6˚C. When stored as directed, the 1X solution is stable for up to 6 months.

1.6 To make a 10X stock solution of the Click-iT™ EdU buffer additive (Component I), add 4 mL of deionized water and mix until 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.

1.7 To make the working solution of the Click-iT™ EdU CellCycle 633-red (Component J, included with Cat. nos. A10034, C35002), add 125 µL DMSO (Component C) to Component J 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.

Note: Component J from Cat. no A10202 is supplied as a working solution and does not need to be reconstituted.

1.8 To make the stock solution of the Click-iT™ EdU CellCycle 488-red (7-AAD, Component K, included with Cat. nos. A10034, C35002), add 125 µL DMSO (Component C) to Component K 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.

Note: Component K from Cat. no A10202 is supplied as a working solution and does not need to be reconstituted.

Experimental Protocols

Labeling Cells with EdU

The following protocol was developed with Jurkat cells, a human T cell line, and 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 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 cells in appropriate tissue culture medium to obtain optimal conditions for cell growth. Try not to disturb cells too much prior to incubation with EdU (*i.e.*, temperature changes, washing) as this 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 longer incubations, *i.e.*, >24 hours, we recommend lower concentrations. For shorter incubations, *i.e.*, ≤30–45 minutes, you can use higher concentrations. 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 depends on the cell growth rate.

2.4 Harvest cells and proceed immediately to **Cell Fixation and (optional) Antibody Cell-Surface Staining,** or fix cells by following steps 3.1–3.3 followed by steps 3.8–3.10, and adding 100 µL of 1% BSA in PBS to each tube. You can store the fixed cells at 4˚C for up to one week. Following storage, process the labeled cells starting with step 4.1 or 5.1.

Cell Fixation and (optional) Antibody Cell-Surface Staining

3.1 After harvesting cells, wash cells once with 1% BSA in PBS, pellet cells by centrifugation at 500 × *g* for 5 minutes, and remove supernatant.

3.2 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 If performing antibody cell-surface labeling proceed to step 3.5. If not, proceed to step 3.8.

3.5 Add surface antibodies and mix well (see Table 2).

3.6 Incubate for the recommended time and temperature or on ice, **protected from light.**

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

3.8 Add 100 µL Click-iT™ fixative (Component D) to the flow tubes. Dislodge the pellet and mix well to ensure a homogenous sample.

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

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

3.11 Proceed to **Cell Permeabilization and EdU Detection** and the optional intracellular staining procedure, step 4.1 or step 5.1.

Cell Permeabilization and EdU Detection

This kit includes two permeabilization reagents:

- Use the Click-iT™ saponin-based permeabilization and wash reagent (prepared in step 1.4) 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 the leukocytes while lysing the red blood cells. We recommend the Click-iT™ saponin-based reagent as the default permeabilization reagent. If using this permeabilization reagent, proceed to step 4.1.
- Use the Click-iT™ Triton® X-100-based permeabilization reagent (Component F) as an alternative to the saponin-based permeabilization reagent. You may use this reagent on cell suspensions if Triton® X-100 has already been validated as the permeabilization reagent with the antibodies utilized to identify intracellular antigens. If using this permeabilization reagent, proceed to step 5.1.

Table 3. Click-iT™ reaction cocktails.

Reaction components	Number of reactions						
	1	2	5	10	15	30	50
1X Click-iT™ Reaction Buffer (prepared in step 1.5)	438 µL	875 µL	2.19 mL	4.38 mL	6.57 mL	13.2 mL	21.9 mL
$CuSO_4$ (Component H)	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 4.6)	50 µL	100 µL	250 µL	500 µL	750 µL	1.5 mL	2.5 mL
Total volume	500 µL	1 mL	2.5 mL	5 mL	7.5 mL	15 mL	25 mL

Saponin-Based Permeabilization, Intracellular Staining (optional), and EdU Detection

4.1 Dislodge the cell pellet prepared in step 3.10 and mix well to ensure a homogenous sample. Add 100 µL of the 1X saponin-based permeabilization and wash buffer prepared in step 1.4, and mix well.

4.2 *Optional*: Add antibodies against intracellular antigens and mix (see Table 2).

4.3 Incubate for the recommended time and temperature, **protected from light**.

4.4 Wash each tube with 3 mL of the 1X saponin-based permeabilization and wash reagent, pellet cells, and remove supernatant. Dislodge the pellet and mix well prior to adding the Click-iT™ reaction cocktail.

4.5 If using red blood cell suspensions, repeat wash step 4.4 once. Take care to remove all residual red blood cells before proceeding to step 4.6.

4.6 Prepare 1X Click-iT™ EdU buffer additive by diluting the 10X solution (prepared in step 1.6) 1:10 in deionized water.

4.7 Prepare Click-iT™ reaction cocktail according to Table 3.

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

4.8 Add 0.5 mL Click-iT™ reaction cocktail (prepared in step 4.7) to each tube and mix well.

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

4.10 Wash once with 3 mL 1X saponin-based permeabilization and wash reagent (prepared in step 1.4), pellet cells, and remove supernatant. Dislodge the pellet and mix.

4.11 Add 0.5 mL of 1X saponin-based permeabilization and wash reagent (prepared in step 1.4).

4.12 If measuring DNA content and cell cycle distribution, proceed to step 6.1. To analyze cells by flow cytometry, proceed to step 7.1.

5.1 Dislodge the cell pellet prepared in step 3.10 and mix well to ensure a homogenous sample. Add 100 µL of the Triton® X-100-based permeabilization reagent (Component F), and mix well.

5.2 *Optional*: Add antibodies against intracellular antigens and mix well (see Table 2).

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

5.4 Wash each tube with 3 mL of 1% BSA in PBS, pellet cells, and remove supernatant. Dislodge the pellet and mix well to ensure a homogenous sample prior to proceeding to step 5.5.

5.5 Prepare the Click-iT™ reaction cocktail according to Table 2.

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

5.6 Add 0.5 mL of Click-iT™ reaction cocktail (prepared in step 5.5) to each tube and mix well.

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

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

5.9 Add 0.5 ml of 1% BSA in PBS.

5.10 If measuring DNA content and cell cycle distribution, proceed to step 6.1. To analyze samples by flow cytometry proceed to step 7.1.

Optional: Measuring DNA Content and Cell Cycle Distribution

This kit includes two stains for measuring DNA content and cell cycle distribution (see Table 4). For a given experiment, only one is used.

Note: You can use propidium iodide (PI) for cell cycle analysis with Alexa Fluor® 488 dye; however, this may reduce the Click-iT™ Alexa Fluor® 488 EdU signal to background ratio.

6.1 Add 5 µL of Ribonuclease A (Component L) to each tube and mix unless you are using CellCycle 405-blue. If using CellCycle 405-blue, proceed directly to step 6.2.

6.2 Add 2 µL of the appropriate cell cycle dye to each tube, mix well, and incubate at room temperature (see Table 4 for details).

Table 4. Click-iT™ EdU CellCycle stains and reaction parameters.

CellCycle stain	EdU detection	Cell cycle		
		Incubation	Excitation	Detection
CellCycle 488-red (7-AAD)	Alexa Fluor® 488 or Pacific Blue™	30 minutes	488 nm	Red emission filter (660/20 nm or similar)
CellCycle 633-red	Alexa Fluor® 488 or Pacific Blue™	15 minutes	633/635 nm	Red emission filter (670/14 nm or similar)
CellCycle 405-blue	Alexa Fluor® 647	30 minutes	405 nm	Blue emission filter (450/50 nm or similar)
Propidium iodide (PI)	Alexa Fluor® 647	30 minutes	488 nm	Red emission filter (610/20 nm or similar)

Note: The complete excitation and emission spectra for these fluorophores are available at www.invitrogen.com/spectraviewer.

Analysis by Flow Cytometry If measuring total DNA content, use a low flow rate during acquisition.

7.1 For the detection of EdU with Alexa Fluor® 488 azide, use 488 nm excitation with green emission filter (*i.e.*, 530/30 nm or similar). The fluorescent signal generated by the Alexa Fluor® 488 azide is best detected with logarithmic amplification.

For the detection of EdU with Pacific Blue™ azide, use 405 nm excitation with violet emission filter (*i.e.*, 450/50 nm or similar). The fluorescent signal generated by the Pacific Blue™ azide is best detected with logarithmic amplification.

For the detection of EdU with Alexa Fluor® 647 azide, use 633/635 nm excitation with red emission filter (*i.e.*, 660/20 nm or similar). The fluorescent signal generated by the Alexa Fluor® 647 azide is best detected with logarithmic amplification.

7.2 For the detection of DNA content:

- Click-iT™ EdU CellCycle 633-red—red emission filter (*i.e.*, 670/14 nm or similar)
- Click-iT™ EdU CellCycle 488-red—red emission filter (*i.e.*, 660/20 nm or similar)
- Click-iT™ EdU CellCycle 405-blue—blue emission filter (*i.e.*, 450/50 nm or similar)
- Propidium iodide (PI)—red emission filter (*i.e.*, 610/20 nm or similar)

The fluorescent signal generated by the CellCycle stains and propidium iodide is best detected with linear amplification.

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).

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

Cat no.	Product Name	Unit Size
A10034	Click-iT™ EdU Pacific Blue™ Flow Cytometry Assay Kit *50 assays*	1 kit
A10202	Click-iT™ EdU Alexa Fluor® 647 Flow Cytometry Assay Kit *50 assays*	1 kit
C35002	Click-iT™ EdU Alexa Fluor® 488 Flow Cytometry Assay Kit *50 assays*	1 kit
Related products		
A1310	7-aminoactinomycin D (7-AAD)	1 mg
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
A10044	EdU (5-ethynyl-2´-deoxyuridine)	50 mg
A10208	Click-iT™ EdU Alexa Fluor® 647 High-Throughput Imaging (HCS) Assay *2-plate size*	1 kit
A10209	Click-iT™ EdU Alexa Fluor® 594 High-Throughput Imaging (HCS) Assay *2-plate size*	1 kit
H1399	Hoechst 33342, trihydrochloride, trihydrate	100 mg
H3570	Hoechst 33342, trihydrochloride, trihydrate *10 mg/mL solution in water*	10 mL
H21492	Hoechst 33342, trihydrochloride, trihydrate *FluoroPure™ grade*	100 mg
V35003	Vybrant® DyeCycle™ Violet stain *5 mM in water* *200 assays*	200 µL
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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