Click-iT® EdU Plus for Flow Cytometry: Enhanced Compatibility for Multiplexing

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ABSTRACT

The Click-iT® EdU proliferation technology is a faster, more reliable cell proliferation method than the well established technology, the bromo-deoxyuridine (BrdU) assay. However, for all of its success, the technology was limited in its multiplexing capability when combined with fluorescent proteins due to the toxic effects of reactive oxygen species and copper quenching. The new Click-iT® EdU Plus kits for S-phase determination can now be run simultaneously with GFP and R-PE as well as other fluorescent proteins and phycobiliproteins. The new Click-iT® EdU Plus kits demonstrate the development of key process innovations of the current kit to make a "copper safe" click reaction. The new Click-iT® EdU Plus kits allow for the ability to multiplex cell proliferation with fluorescent protein expression as well as with immunophenotyping using the most popular fluorochromes.

The protection of R-PE, R-PE tandem dyes, and GFP were tested on Jurkat cells. A Click-iT® EdU Plus assay was performed and the cells were stained with a cell cycle dye before being analyzed on an Attune® acoustic-focusing flow cytometer. Controls of the click and fluorescent protein signals were compared to the performance of the new Click-iT® EdU Plus assay. Three modified dye azides—Alexa Fluor® 488, Alexa Fluor® 647, and Pacific Blue™—were tested with an appropriate cell cycle dye.

In every click reaction tested, the new Alexa Fluor® 488, AlexaFluor® 647 and Pacific Blue™ Click-iT® Plus EdU assay provides a significantly brighter EdU signal than the existing Click-iT® EdU assay. When tested with the new Click-iT® EdU Plus flow cytometry assays, 60-80% of the GFP, R-PE and R-PE tandem fluorescent protein signal was protected when compared to the no copper control.

With the modifications to the click reaction the new assay is capable of multiplexing with many fluorochromes. The new Click-iT® EdU Plus products will allow researchers to multiplex a cell proliferation assay with many fluorochromes that weren't able to be used with the current Click-iT® EdU products now available. Click-iT® Plus EdU assays deliver the most rapid and most sensitive proliferation kits with the highest degree of compatibility with a broad range of commonly used fluorochromes such as R-PE, R-PE tandems, Brilliant Violet™ Dyes, and fluorescent proteins.

INTRODUCTION

Cell proliferation assays are designed to detect nascent DNA synthesis. In the Click-iT® Plus EdU proliferation assay, an alkyne-containing thymidine analog, 5-ethynyl-2´-deoxyuridine, (EdU) is incorporated into DNA during active DNA synthesis (1). The newly synthesized DNA is then detected by reaction of the incorporated EdU with an azide containing fluorescent dye using a copper-catalyzed azide–alkyne cyloaddition-- the so-called "click" reaction (1). The click reaction utilizes a relatively small dye for detection, instead of a large antibody conjugate, enabling efficient detection of synthesizing DNA without the need for chromatin denaturation. Additionally, the reaction is quick, typically completed in 60 min, and robust. The new Click-iT® Plus technology builds upon the success of the already popular Click-iT® labeling strategy by utilizing a picolyl azide fluorescent dye that reacts with chelated copper, and a copper protectant to produce the same sensitive, reliable detection of cell proliferation as in the previous version of the assay. With the picolyl azide and the copper chelating approach, Click-iT® Plus assays can be used to accurately determine cell proliferation while preserving cell morphology, DNA integrity, antigen binding sites, and the fluorescence of proteins such as R-PE and GFP.

MATERIALS AND METHODS

Click-iT® Plus EdU and fluorophores

Jurkat (human T-cell leukemia) cells were grown to confluency and treated for 2 hours with 10uM EdU. Cells were then harvested, washed with 1x PBS and resuspended in 1x PBS at 10^6 cells/ml. The cells were then stained with a fluorophores for 20 minutes in the dark at room temperature, washed with 1x PBS and resuspended in 100uL per 10^6 cells of component D from the Click-iT® Plus EdU flow cytometry assay kit. A click assay was then carried out according to the manufacturer's instructions and an appropriate fixable cell cycle stain was used according the manufacturing and, finally, cells were analyzed on an Attune® acoustic-focusing flow cytometer.

A375 (human malignant melanoma) cells, which express Green Fluorescent Protein (GFP) were grown to confluency and treated with 10uM EdU like the Jurkat cells, but as they didn't require antibody-conjugate staining, they were washed, resuspended, then a click assay was performed as in the Jurkat cells.

Click-iT® Plus EdU and PE-phospho-histone H3

Jurkat (human T-cell leukemia) cells were treated with 500uM nocodazole for 18 hours and then treated for 2 hours with 10uM EdU. Cells were harvested, washed with 1x PBS and resuspended in 100uL per 10^6 cells of component D from the Click-iT® Plus EdU flow cytometry assay kit. Cells were incubated for 15 minutes in dark at room temperature, washed with 1x PBS and resuspended in 100uL per 10^6 cells of component E from the Click-iT® Plus EdU flow cytometry assay kit. The cells were then stained with PE-phospho-histone H3 antibody conjugate (Ser10) (D2C8), an intracellular marker, for 20 minutes in the dark at room temperature. A click assay was then carried out according to the manufacturer's instructions and an appropriate fixable cell cycle stain was used according the manufacturing and, finally, cells were analyzed on an Attune® acoustic-focusing flow cytometer.

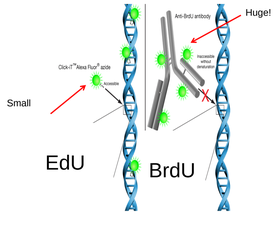

Figure 1: Schematic of EdU and BrdU Incorporation into DNA

RESULTS

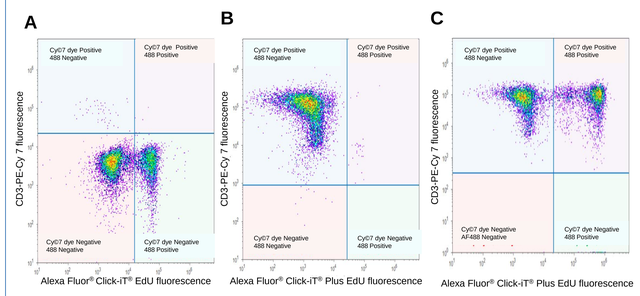

Figure 2: Alexa Fluor® 488 Click-iT® Plus EdU Flow Cytometry Assay with CD3-PE Cy©7
Jurkat (human T-cell leukemia) cells were treated with 10 µM EdU for 2 hours, stained with CD3-PE Cy©7 dye (Cat. No MHCD0312) and analyzed according to the recommended staining protocol. **Panel A,** dual parameter plot of cells labeled with the classic Click-iT® EdU Alexa Fluor® 488 picolyl azide and CD3-PE. **Panel B,** dual parameter plot of cells labeled with the Click-iT® EdU Plus Alexa Fluor® 488 picolyl azide minus copper and CD3-PE Cy©7 dye. **Panel C,** dual parameter plot of cells labeled with the Click-iT® EdU Plus Alexa Fluor® 488 picolyl azide and CD3-PE Cy©7 dye.

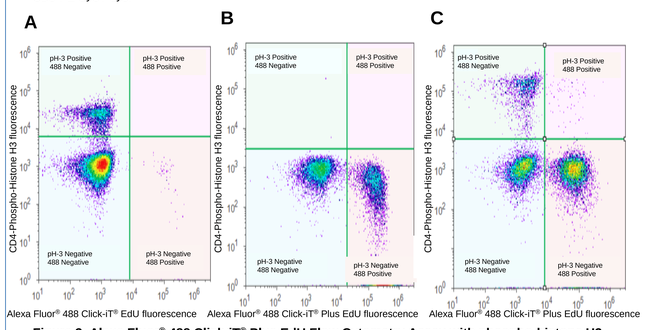

Figure 3: Alexa Fluor® 488 Click-iT® Plus EdU Flow Cytometry Assay with phospho-histone H3
Jurkat (human T-cell leukemia) cells were treated with 500uM nocodozale for 18 hours, then treated with 10 µM EdU for 2 hours, stained with CD4 conjugated to phospho-histone H3 and analyzed according to the recommended staining protocol. **Panel A,** dual parameter plot of cells labeled with the classic Click-iT® EdU Alexa Fluor® 488 picolyl azide and CD4-phospho-histone H3. **Panel B,** dual parameter plot of cells labeled with the Click-iT® EdU Plus Alexa Fluor® 488 picolyl azide minus copper and CD4-phospho-histone H3. **Panel C,** dual parameter plot of cells labeled with the Click-iT® EdU Plus Alexa Fluor® 488 picolyl azide and CD4-phospho-histone H3.

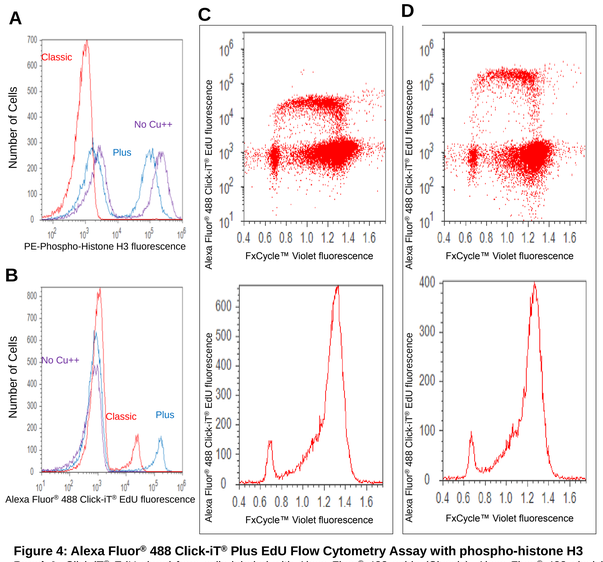

Figure 4: Alexa Fluor® 488 Click-iT® Plus EdU Flow Cytometry Assay with phospho-histone H3
Panel A, Click-iT® EdU signal from cells labeled with Alexa Fluor® 488 azide (Classic), Alexa Fluor® 488 picolyl azide (Plus), or a no copper control (No Cu++), all co-stained with PE-phopsho-histone H3 after 18 hour treatment with 500uM nocodazole. **Panel B,** click fluorescence signal from the same cells. **Panel C,** dual parameter plot of PE-phospho-histone H3 stained cells labeled with FxCycle™ Violet and the classic Click–iT® EdU reaction (upper); cell cycle alone (lower). **Panel D,** dual parameter plot of PE-phospho-histone H3 stained cells labeled with FxCycle™ Violet and the Click-iT® EdU Plus reaction (upper); cell cycle alone (lower).

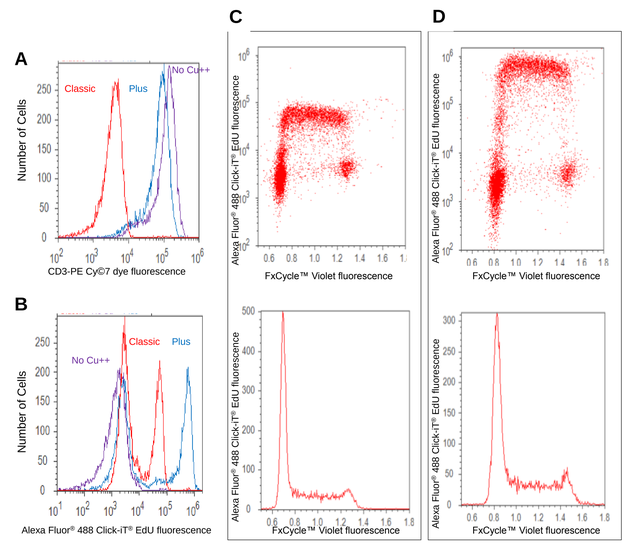

Figure 5: Alexa Fluor® 488 Click-iT® Plus EdU Flow Cytometry Assay and CD3-PE Cy©7 dye
Panel A, CD3-PE Cy©7 dye signal from cells labeled with Alexa Fluor® 488 azide (Classic), Alexa Fluor® 488 picolyl azide (Plus), or a no copper control (No Cu++), all co-stained with CD3-PE Cy©7 dye. **Panel B,** click fluorescence signal from the same cells. **Panel C,** dual parameter plot of CD3-PE Cy©7dye -stained cells labeled with FxCycle™ Violet and the classic Click–iT® EdU reaction (upper); cell cycle alone (lower). **Panel D,** dual parameter plot of CD3-PE Cy©7 dye-stained cells labeled with FxCycle™ Violet and the Click-iT® EdU Plus reaction (upper); cell cycle alone (lower).

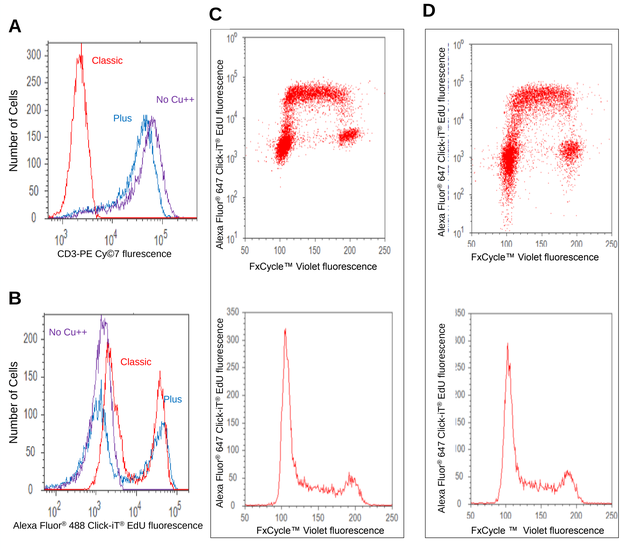

Figure 6: Alexa Fluor® 647 Click-iT® Plus EdU Flow Cytometry Assay and CD3-PE Cy©7
Panel A, CD3-PE signal from cells labeled with Alexa Fluor® 647 azide (Classic), Alexa Fluor® 647 picolyl azide (Plus), or a no copper control (No Cu++), all co-stained with CD3-PE. **Panel B,** click fluorescence signal from the same cells. **Panel C,** dual parameter plot of CD3-PE-stained cells labeled with FxCycle™ Violet and the classic Click –iT® EdU reaction (upper); cell cycle alone (lower). **Panel D,** dual parameter plot of CD3-PE-stained cells labeled with FxCycle™ Violet and the Click-iT® EdU Plus reaction (upper); cell cycle alone (lower).

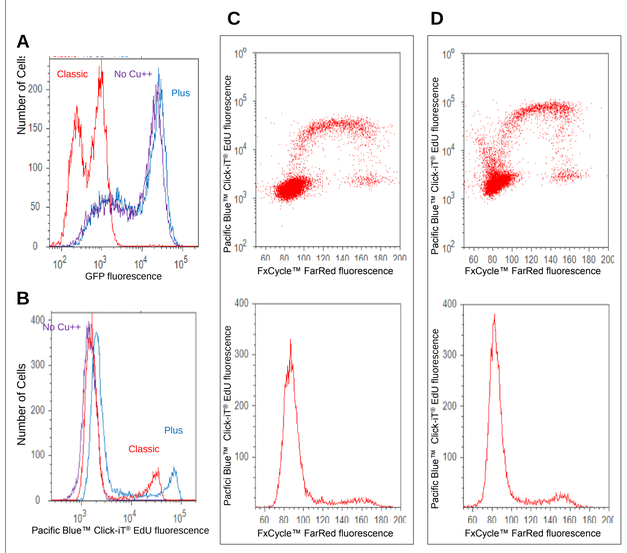

Figure 7: Pacific Blue® Click-iT® Plus EdU Flow Cytometry Assay and GFP
Panel A, GFP signal from cells labeled with Pacific Blue® azide (Classic), Pacific Blue™ picolyl azide (Plus), or a no copper control (No Cu++), all with GFP-expressing cells. **Panel B,** click fluorescence signal from the same cells. **Panel C,** dual parameter plot of GFP-expressing cells labeled with FxCycle™ FarRed and the classic Click –iT® EdU reaction (upper); cell cycle alone (lower). **Panel D,** dual parameter plot of GFP-expressing cells labeled with FxCycle™ FarRed and the Click-iT® EdU Plus reaction (upper); cell cycle alone (lower).

	Click-iT® EdU	Click-iT® Plus EdU
Workflow	3 hours	<3 hours
R-PE compatible	post-click staining	YES
R-PE tandem compatible	post-click staining	YES
PerCP compatible	YES	YES
APC compatible	YES	YES
GFP compatible	NO	YES
mCherry compatible	YES	YES
polymer dye compatible	YES	YES

Table 1: Click-iT® Plus EdU functionality chart compared to classic Click-iT® EdU

CONCLUSIONS

The new Click-iT® EdU Plus kits demonstrate the chemistry prowess of Molecular Probes® with the development of key process innovations in the components of the current kit to make a "copper safe" click reaction. These process innovations have led to a cell proliferation assay kit that delivers the most rapid and robust results with the highest degree of compatibility with commonly used fluorochromes.

REFERENCES

1. Salic A, Mitchison TJ (2008) *Proc Natl Acad Sci USA* 105, 2415-2420.

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