

Detection of S-phase cell cycle progression using 5-ethynyl-2'-deoxyuridine incorporation with click chemistry, an alternative to using 5-bromo-2'-deoxyuridine antibodies

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The 5-bromo-2'-deoxyuridine (BrdU) labeling of cells followed by antibody staining has been the standard method for direct measurement of cells in the S-phase. Described is an improved method for the detection of S-phase cell cycle progression based upon the application of click chemistry, the copper(I)-catalyzed variant of the Huisgen [3+2] cycloaddition between a terminal alkyne and an azide. 5-ethynyl-2'-deoxyuridine (EdU) is a nucleoside analog of thymidine that is incorporated into DNA during active DNA synthesis, just like BrdU. While the BrdU assay requires harsh chemical or enzymatic disruption of helical DNA structure to allow for direct measurement of cells in the S-phase by the anti-BrdU antibody, the EdU method does not. Elimination of this requirement results in the preservation of helical DNA structure and other cell surface epitopes, decreased assay time, and increased reproducibility.

Labeling, detection, and quantification of cells actively synthesizing DNA, or cells in the S-phase of cell cycle progression, are not only important in characterizing the basic biology but also in defining the cellular responses to drug treatments, assessing cell health, and determining genotoxicity. The history (1,2) of S-phase cell cycle detection and measurement began in the 1960s with the incorporation of radioactive nucleosides to detect DNA synthesis. The use of autoradiography for analysis was slow and technically difficult. It was subsequently replaced with antibody-based detection of the nucleoside analog 5-bromo-2'-deoxyuridine (BrdU) as the standard method for direct measurement of cells in the S-phase (3). Although an improvement over the older techniques, the typical BrdU assay has limitations. The protocols often require the harsh treatment of DNA using a combination of acid, heat, or nucleases to allow the anti-BrdU antibody access to the incorporated BrdU residues. This harsh treatment results in the loss of helical DNA structure required for cell cycle dye binding and destroys many

cellular antibody epitopes. Obtaining the fine balance required for simultaneous antibody detection and cell cycle staining is very time-consuming and in some cell types, technically difficult to achieve. To address these limitations, numerous protocols have been developed over the years using the BrdU antibody detection method (4).

Described here is an improved cell proliferation assay for the detection of S-phase cell cycle progression based upon the incorporation of 5-ethynyl-2'-deoxyuridine (EdU) instead of

BrdU. Detection of the newly incorporated nucleotide analog is achieved via reaction of the ethynyl group with a small fluorescent azide-containing probe (Click-iT EdU Alexa Fluor Cell Proliferation Assay kit; Invitrogen, Carlsbad, CA, USA) in a click reaction (Figure 1). The click reaction was first described by the Sharpless and Meldal groups independently in 2002 (5,6), and has proven to be a powerful new ligation chemistry. The small size of the reaction components obviates the need for harsh DNA treatment, thus maintaining the helical structure required for cell cycle staining and antibody epitopes for labeling other cell surface markers. This phenomenon underscores the fact that small molecules (0.6 kDa) have easier access to incorporated nucleotides in double-stranded DNA as compared with a larger macromolecule such as an antibody (150 kDa). The limitations of the BrdU protocol have now been eliminated; the improved EdU cell proliferation protocol is highly reproducible and provides an easy, rapid workflow.

The standard EdU DNA proliferation assay requires 1.5 to 2 h after the incorporation of EdU (10 μ M), as compared with traditional anti-BrdU methods requiring a minimum of 4 h to several days. After metabolic labeling with EdU, the cells are fixed, permeabilized, and click-labeled with an azide dye of choice for 30 min in the presence of copper(II) sulfate and a reducing agent to afford the copper(I) that catalyzes the click reaction (7). Although copper(I) is toxic to live cells, copper toxicity is not an issue

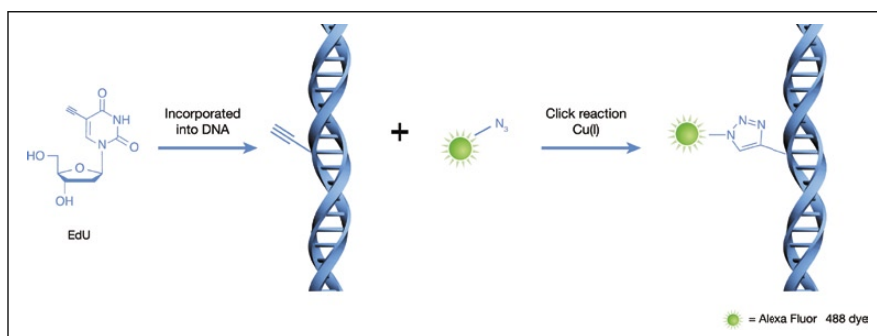


Figure 1. The click reaction between the ethynyl group of the incorporated EdU in the double-stranded DNA and the Alexa Fluor 488 azide. The illustration represents the formation of the triazole bond between the alkyne and the azide; the actual orientation of the alkyne within DNA is unknown.

Benchmarks

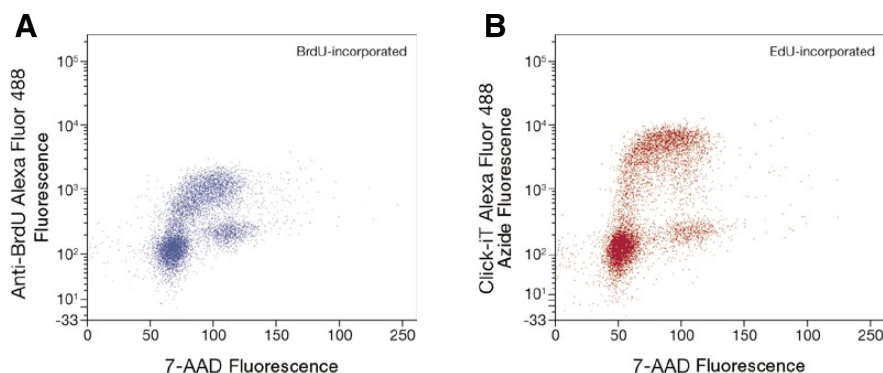


Figure 2. Comparison of EdU detection with BrdU by flow cytometry. (A) Standard antibody method (11). Dual parameter plot of Jurkat (human T cell leukemia) cells labeled with anti-BrdU Alexa Fluor 488 conjugate and 7-amino actinomycin D (7-AAD). Jurkat cells were treated with 10 μ M BrdU for 1 h and tested using the acid denaturation method for BrdU detection, co-labeled with 7-AAD. BrdU-incorporated cells were alcohol-fixed for 3 days at 4°C, washed twice with BSA/PBS buffer, treated with 4N HCl for 20 min, washed twice with a citrate phosphate buffer, resuspended in buffer before staining with anti-BrdU Alexa Fluor 488 dye, and washed again with buffer before staining of DNA content with 7-AAD. The dual parameter plot shows DNA content labeling with the labeling of proliferating cells that have incorporated BrdU; co-positive cells give 36% proliferating cells. (B) New click method. Dual parameter plot of Jurkat cells labeled with Click-iT Alexa Fluor 488 azide and 7-AAD. Jurkat cells were treated with 10 μ M EdU for 1 h. EdU-incorporated cells were fixed with paraformaldehyde for 15 min, washed once with BSA/PBS buffer, permeabilized with a saponin-based buffer for 30 min, washed once, treated with the click-reaction mixture for 30 min, washed once, and resuspended in buffer before staining of DNA content with 7-AAD. Cells were analyzed on the LSRII Flow Cytometer (BD Biosciences, San Jose, CA, USA). The dual parameter plot shows DNA content labeling with the labeling of proliferating cells that have incorporated EdU; co-positive cells give 35% proliferating cells.

because the cells are fixed and permeabilized before the detection reaction is performed. If multiplexed staining of cell surface markers is desired, labeled antibodies of interest can be added as per standard protocols before the cell fixation step. Fluorescence readout can be performed using various methods such as flow cytometry and fluorescence microscopy. Side-by-side comparison of the new EdU and standard BrdU assays demonstrates that the new click EdU methodology provides the comparable sensitivity of detection as that obtained from the BrdU protocols (Figure 2). Although the comparative efficiencies of intracellular EdU and BrdU as phosphorylation and polymerase substrates have not been directly determined, the fact that the loading doses and the final assay sensitivities using each nucleoside are directly proportionate suggests that they are equivalently processed in vivo.

Key to the low background and high sensitivity of the EdU assay is the click reaction, which involves the coupling of an azide-labeled compound and a terminal alkyne-labeled compound in the presence of copper(I) to form a stable covalent triazole ring conjugate. Because the click-reaction components are not normally found in biological systems, the reaction is bioorthogonal and can proceed in the presence of complex biological mixtures, resulting in high selectivity and low background in the detection reaction. The copper(I) required to catalyze the reaction can be generated via the reduction of copper(II) to copper(I) in situ using various reducing agents or alternatively, copper(I) can be added directly into the reaction mix in the form of copper bromide (CuBr) or copper wire (8). Copper chelators and ligands have been used in attempts to protect proteins and virus particles from damage caused by copper toxicity (9,10). Regardless of the methods used, exposure of live cells to copper(I) or copper(II), with or without chelators or ligands, results in marked cell death in a short period of time. For the EdU assay, exposure of copper to cells occurs after fixation and permeabilization and therefore has no effect on cell viability.

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In summary, the method detailed above provides superior labeling, detection, and quantification of cells actively synthesizing DNA. The ability to detect the incorporated EdU in helical DNA is a vast improvement over the standard BrdU protocol because it does not require the problematic, harsh treatment of DNA. The technique preserves helical DNA structure for simultaneous staining with cell cycle dyes and preserves cell surface antibody epitopes for multiplexed high-throughput analyses. In addition, assay reproducibility is increased and assay time is greatly reduced. Finally, the EdU assay remains as cost effective as the standard BrdU protocol.

COMPETING INTERESTS STATEMENT

S.B., J.B., K.G., B.A., and S.C. are employed by Molecular Probes Detection Technologies, Invitrogen Corporation, the company that sells the reagents for the assay described in this article. A.S. declares no competing interests.

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