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Click-&-Go™ EdU 647 Flow Cytometry Assay Kit, 50 assays

Product No. 1391

Introduction

The Click-&-Go EdU 647 Flow Cytometry Assay Kit is a superior alternative to the traditional cell proliferation assays that use radioactive nucleosides, i.e., 3H-thymidine or antibody-based detection of the nucleoside analog bromodeoxyuridine (BrdU). EdU (5-ethynyl-2'-deoxyuridine) is a nucleoside analog of thymidine with a terminal alkyne group that is efficiently incorporated into DNA during active DNA synthesis. In the next step, newly synthesized EdU-labeled DNA is fluorescently labeled through a click reaction with the best-in-class far red fluorescent Azide 647 (Alexa Fluor® 647 Azide replacement). Standard flow cytometry methods are used for determining the percentage of S-phase cells in the population. Sufficient amounts of reagents are provided for 50 assays based on the following protocol.

Kit contents

Component	Concentration	Amount
EdU (Component A)	-	10 mg
Azide 647 (Component B)	-	1 vial
Fixative (Component C)	4% PBS solution	5 mL
Copper (II) Sulfate (Component D)	100 mM	0.55 mL
Reducing Agent (Component E)	-	400 mg
Saponin-based permeabilization and wash reagent (Component F)	10x solution	50 mL

Materials required but not provided

- 1% BSA in PBS (pH 7.4)
- Flow cytometry tubes
- PBS buffer, pH 7.4
- Deionized water
- DMSO

Additional information

- The Fixative (Component C) contains paraformaldehyde, which can be harmful to human health. Use with appropriate precautions.
- The Saponin-based permeabilization and wash reagent (Component F) 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.

Material preparation

EdU (Component A)	Add 2 mL of DMSO to make 20 mM stock solution. This solution is stable for up to 1 year if stored at -20C and protected from light.
Azide 647 (Component B)	Add 140 μL of DMSO or water. This solution is stable for up to 1 year if stored at -20C and protected from light.
1X saponin-based permeabilization and wash reagent (Component F)	To prepare 500 mL of 1X saponin-based permeabilization and wash reagent, add 50 mL of **Component F** to 450 mL of 1% BSA in PBS. Smaller amounts can be prepared by diluting a volume of **Component F** 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.
Reducing Agent (Component E)	Prepare only enough 1 x solution of **Reducing Agent (Component E)** for one day. Weigh out 20 mg of **Reducing Agent (Component E)** into 2 mL vial and add 1 mL of deionized water. Vortex until completely dissolved. Note – The reducing agent is susceptible to oxidation and turns brown when oxidized. Do not use if solution appears brown.

Cell labeling with EdU

This protocol has been successfully tested with HEK 293T, HeLa, and NIH/3T3 cells with an optimized EdU concentration of 10 μM, and it can be easily adapted for any adherent cell type. Growth medium, cell density, cell type variations, and other factors may influence labeling. Investigators may wish to determine the optimal concentration of EdU reagent as well as labeling time individually for each cell type on a small-scale before proceeding. Metabolic labeling is a critical step for successful cell proliferation and should be carefully assessed for each cell line of interest.

1.1 Suspend the cells in an appropriate 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.

1.2 Add the desired amount of EdU to the cells in culture medium to achieve the optimal working EdU concentration (10 µM, if not optimized). We have found a final concentration of 10 µM EdU to be sufficient for labeling of most cell lines.

1.3 During the addition of EdU to the cells in culture, avoid disturbing the cells in ways that may disrupt the normal cell cycling patterns.

1.4 Incubate the cells for the desired length of time under conditions optimal for the cell type. Different cell types may require different incubation periods for optimal labeling with EdU. As a starting point we recommend 10 µM EdU for 1-2 hours.
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.

1.5 Proceed immediately to **Cell fixation and permeabilization** or **cell-surface antigen staining with antibodies (optional).**

Optional cell-surface antigen staining with antibodies

2.1 After EdU labeling, wash cells once with 3 mL of 1% BSA in PBS, pellet cells by centrifugation, and remove the supernatant.

2.2 Dislodge the pellet and resuspend the cells at a concentration of 1×10^7 cells/mL in 1% BSA in PBS.

2.3 Add surface antibodies, mix well, and incubate for the recommended time and temperature, keeping cells protected from light.

2.4 Proceed to cell fixation step.

Cell fixation and permeabilization

The following protocol is provided for the fixation step using a saponin-based permeabilization and wash reagent. This 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. Protocols using other fixation/permeabilization reagents, such as methanol and 3.7% formaldehyde in PBS followed by a 0.5% Triton®X-100 permeabilization step can also be used.

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

3.2 Dislodge the pellet, add 100 µL of Fixative (Component C) and mix well, then incubate for 15 minutes.

3.3 Remove the fixative and wash the cells with 1 mL of 1% BSA in PBS. If red blood cells or hemoglobin are present in the sample, repeat the wash step. If required, remove all residual cell debris.

3.4 Remove the wash solution and resuspend the cells in 100 µL of 1X saponin-based permeabilization and wash reagent, and mix well. Incubate the cells for 15 minutes.

3.5 Proceed directly to the EdU detection step, or to treating cells with antibodies to intracellular antigens followed by the EdU detection step.

EdU detection

Note: 500 µL is used per reaction. A smaller volume can be used as long as the remaining reaction components are maintained at the same ratios.

4.1 Weigh out 20 mg of **Reducing Agent (Component E)** into a 2 mL vial, add 1 mL of deionized water, and vortex until completely dissolved. This solution should be freshly prepared and used on the same day.

4.2 Prepare the required amount of the reaction cocktail according to Table 1. **Add the ingredients in the order listed in the table**. Use the reaction cocktail within 15 minutes of preparation.

Table 1.

Reaction component	Number of reactions					
	1	**2**	**5**	**10**	**15**	**50**
PBS buffer	438 µL	875 µL	2.19 mL	4.38 mL	6.57 mL	21.9 mL
Copper (II) Sulfate (Component D)	10 µL	20 µL	50 µL	100 µL	150 µL	500 µL
Azide 647 solution (Material preparation)	2.5 µL	5 µL	12.5 µL	25 µL	37.5 µL	125 µL
1x Reducing Agent (step 3.1)	50 µl	100 µL	250 µL	500 µL	750 µL	2.5 mL
Total Volume	500 µL	1 mL	2.5 mL	5 mL	7.5 mL	25 mL

4.3 Remove the permeabilization buffer (step 3.5).

4.4 Add 0.5 mL of the **Reaction Cocktail** to each tube. Rock the tube briefly to ensure that the reaction cocktail is distributed evenly.

4.5 **Protect from light**, and incubate the tube for 30 minutes at room temperature.

4.6 Remove the reaction cocktail.
Wash the cells well once with 3 mL of 1X saponin-based permeabilization and wash reagent.
Pellet the cells and remove the wash solution.

4.7 If intracellular antibody labeling is required, dislodge the cell pellet and resuspend the cells in 100 µL of 1X saponin-based permeabilization and wash reagent.

4.8 If no intracellular antibody labeling is required, add 500 µL of 1X saponin-based permeabilization and wash reagent.

At this point the samples are ready for **DNA staining**. If no **DNA staining** is desired, proceed to analyzing the cells on a flow cytometer.

Optional intracellular or surface antigens staining

5.1 Add antibodies against intracellular antigens or against surface antigens that use RPE, PR-tandem or Quantum Dot antibody conjugates. Mix well.

5.2 Protect from light and incubate the tubes for the time and temperature required for antibody staining.

5.3 If **EdU Detection** has not yet been performed, 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 µL of 1x saponin-based permeabilization and wash reagent, mix well and proceed to **EdU Detection** (step 4.1)

5.4 If **EdU Detection** has been performed (steps 4.1–4.8), wash each tube with 3 mL of 1X saponin-based permeabilization and wash reagent, pellet the cells, and remove the supernatant. Dislodge the cell pellet and resuspend the cells in 500 µL of 1X saponin-based permeabilization and wash reagent, and proceed to DNA staining, or to the cell analysis on a flow cytometer.

Optional DNA staining

6.1 Add the appropriate DNA stain to each tube and mix well.

6.2 Protect from light and incubate as recommended by the manufacturer.

6.3 Remove the DNA stain and wash each tube with 3 mL of 1X saponin-based permeabilization and wash reagent.

Flow Cytometry Analysis

If a traditional flow cytometer with a hydrodynamic focusing is used to measure the total DNA content, use a low flow rate during acquisition. The same collection rate and cell concentration should be used for all samples within an experiment. Detect the fluorescent signal generated by DNA content stains with linear amplification. The fluorescent signal generated by labeling is best detected with logarithmic amplification.

The excitation and emission maxima of Azide 647 are 650 nm and 671 nm.