EZClick™ EdU Cell Proliferation/DNA Synthesis Kit (FACS/Microscopy), Red Fluorescence
(Catalog # K946-100; 100 assays; Store at -20°C)

I. Introduction:
All living cells undergo division cycle, a biological process crucial for proliferation and inheritance. Cell-division cycle is a series of events resulting in two daughter cells containing replicas of DNA from the original DNA molecule. DNA replication occurs in the S phase of the cell cycle and involves de novo synthesis of genomic DNA from its precursors. The ability of monitoring detailed characterization of cell cycle and DNA synthesis in proliferating cells is fundamental in basic, and applied immunologic and oncologic studies. Accurate determination of the effect of biologically active reagents on DNA synthesis and cell cycle is of great importance in anti-cancer drug discovery and basic biology. Biovision’s EZClick™ EdU DNA Synthesis Monitoring Kit utilizes a novel approach that relies on incorporation of 5-Edu (5-ethyl-2'-deoxyuridine) as nucleoside analog to thymidine into newly synthesized DNA directly in the cell culture. Incorporation of EdU into genomic DNA in S-phase is detected based on a click reaction between the alkyne moiety of EdU and fluorescent azide. Compared to historically used BrdU, click reaction is carried in mild conditions and flow cytometry/fluorescence microscopy can be used for assessment of proliferating cells in the population. Our kit provides sufficient materials for 100 assays based on the protocol below.

II. Applications:
- Detection of DNA synthesis in proliferating cells and assessment of cell cycle phase
- Screening for genotoxic compounds and effectors of cell division cycle
- Evaluating effects of anti-cancer drugs and genotoxic agents

III. Sample Type:
- Suspension or adherent cell cultures

IV. Kit Contents:

<table>
<thead>
<tr>
<th>Components</th>
<th>K946-100</th>
<th>Cap Code</th>
<th>Part Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>EZClick™ Wash Buffer (10X)</td>
<td>25 ml</td>
<td>NM</td>
<td>K946-100-1</td>
</tr>
<tr>
<td>Fixative Solution</td>
<td>10 ml</td>
<td>WM</td>
<td>K946-100-2</td>
</tr>
<tr>
<td>Permeabilization Buffer (10X)</td>
<td>25 ml</td>
<td>NM/Blue</td>
<td>K946-100-3</td>
</tr>
<tr>
<td>EZClick™ EdU DNA Label (1000X)</td>
<td>10 μl</td>
<td>Clear</td>
<td>K946-100-4</td>
</tr>
<tr>
<td>Copper Reagent (100X)</td>
<td>100 μl</td>
<td>Blue</td>
<td>K946-100-5</td>
</tr>
<tr>
<td>EZClick™ Fluorescent Azide (100X)</td>
<td>100 μl</td>
<td>Red</td>
<td>K946-100-6</td>
</tr>
<tr>
<td>Reducing Agent (20X)</td>
<td>500 μl</td>
<td>Yellow</td>
<td>K946-100-7</td>
</tr>
<tr>
<td>EZClick™ Total DNA Stain (1000X)</td>
<td>10 μl</td>
<td>Green</td>
<td>K946-100-8</td>
</tr>
</tbody>
</table>

V. User Supplied Reagents and Equipment:
- Tissue culture vessels and appropriate culturing media; flow cytometry vessels
- Phosphate Buffered Saline (PBS, pH 7.4)
- Sterile 0.1% Gelatin Solution (optional, only required for adhering suspension cells to the surface)
- Flow cytometer equipped with laser capable of excitation at 488 and 530/590 nm emission filters respectively
- Fluorescence microscope capable of excitation and emission at 440/490 and 540/580 nm respectively

VI. Storage Conditions and Reagent Preparation:
Upon arrival, store the entire kit at -20°C protected from light. Briefly centrifuge small vials prior to opening. Read the entire protocol before performing the assay.
- 10X Wash Buffer and 10X Permeabilization Buffers: Dilute the 10X stocks 1:10 in sterile water, mix well. Store at 4 °C.
- Fixative Solution: Ready to use, after opening store at 4°C, protected from light.
- Remaining components: Store at -20°C protected from light. While in use, keep on ice and minimize light exposure.

VII. Assay Protocol:
Notes:
This assay was developed with HeLa (adherent) and Jurkat (suspension) cells and can be modified for any cell line. The protocol below refers to a 96-well tissue culture plate; adjust volumes accordingly for other plate formats. The assay volume is 100 μl. Growth conditions, cell number per well and other factors may affect the incorporation rate of the DNA Label; therefore optimize the assay for your cell type. We suggest an initial test of several EZClick™ EdU DNA Label concentrations to find best conditions for tested cell type and experimental design. Avoid stressing the cells by washes or temperature changes prior to incubation with EZClick™ EdU DNA Label. All steps should be carried out at room temperature (RT) unless otherwise specified; equilibrated all buffers to RT prior to the experiment.

1. Labeling of control and experimental cells:
   a. Obtain cell suspension of desired density and seed directly into tissue culture vessels, or on coverslips for high resolution microscopy. To immobilize suspension cells for microscopy: add 100 μl of 0.1% gelatin solution directly into the wells, tilt the plate to cover the entire well surface and place it in a tissue culture hood for 1 hour. Gently remove the gelatin solution and seed your cells. Allow the cells to recover overnight before the treatment.
   b. Next day, remove the media and treat the cells with appropriate effectors according to your protocol; do not add treatment to the positive and negative control cells. Negative control (cells not exposed to the DNA Label or treatment), positive control (cells incubated with 1X EZClick™ EdU DNA Label only).
c. Dilute EZClick™ EdU DNA Label (1000X) to 1X final concentration with culture medium and add into the experimental and positive control cells respectively. Incubate for 0.5 - 24 hours in the 37°C incubator, or for the period of time required by your experimental protocol. For longer incubation, decrease the concentration of the DNA label, or for shorter incubation times, increase the amount.

d. Terminate the experiment by removal of the culture medium and rinse the cells once with 100 µl of PBS, discard the supernatant. For immobilized suspension cells: Centrifuge the plate at 300 x g (or the lowest centrifuge setting) for 5 minutes to deposit the cells onto the surface. Tilt the plate and gently remove the media with a pipette tip. It is important to avoid excessive centrifugation speeds, which can damage the cells. Make note of the place that is used, and perform subsequent aspirations from the same place. Proceed to the Fixation and Permeabilization. Pellet the suspension cells at 300 x g for 5 min throughout the entire protocol!

2. Fixation and Permeabilization:
   a. For adherent cells: Add 100 µl of Fixative Solution to each well and incubate the cells for 15 min at RT protected from light. Remove the fixative and wash the cells twice with 100 µl of 1X Wash Buffer, remove the wash. Add 100 µl of 1X Permeabilization Buffer and incubate the cells for 10 min at RT. Remove the Permeabilization Buffer and replace it with a 20 µl of fresh aliquot.
   b. For suspension cells: Re-suspend the cells in 100 µl of Fixative Solution and incubate for 15 min at RT protected from light. Remove the fixative and wash the cells twice with 0.5 ml of 1X Wash Buffer. Discard the supernatant and re-suspend the cells in 100 µl of 1X Permeabilization Buffer. Incubate the cells for 10 min at RT. Remove the Permeabilization Buffer and replace it with a 20 µl of fresh aliquot. Proceed to EZClick™ reaction and total DNA staining.

3. EZClick™ DNA reaction and total DNA staining:
   a. Reaction cocktail: Prepare 1X EZClick™ reaction cocktail according to the table below. Volumes should be multiplied by number of samples and reagents added in the exact order. Use the reaction cocktail within 15 minutes of preparation. Cells should be protected from light during, and following the EZClick™ reaction and DNA staining.

<table>
<thead>
<tr>
<th>Amount per Reaction</th>
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<tbody>
<tr>
<td>PBS 93 µl</td>
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<tr>
<td>Copper Reagent (100X) 1 µl</td>
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<tr>
<td>EZClick™ Fluorescent Azide (100X) 1 µl</td>
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<tr>
<td>Reducing Agent (20X) 5 µl</td>
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   b. EZClick™ Reaction: Add 100 µl of 1X EZClick™ Reaction cocktail to each sample and incubate the cells for 30 min at room temperature protected from light. Remove the reaction cocktail and wash the cells three times in 100 µl of Wash Buffer. Proceed to DNA staining. If no DNA staining is desired, proceed to Microscopic or FACS analysis. DNA staining: Prepare 1X dilution of EZClick™ Total DNA Stain and add 100 µl per well. Incubate the cells for 20 minutes at room temperature, or re-agitate at 4 °C protected from light. Remove the stain solution; wash the cells once with 100 µl of PBS. Note: cells are compatible with all methods of slide preparation including wet mount or prepared mounting media.

4. Fluorescence Microscope Imaging: Analyze samples for red fluorescence generated by labeled DNA and green by total DNA respectively. FACS analysis: Harvest the cells by preferred method and wash with 0.5 ml of ice-cold PBS. Re-suspend the pellets in 100 µl of ice-cold PBS. Transfer the cell suspension into flow cytometry vessels. Analyze samples in FL-2 channel for signal generated by DNA during click reaction. Note: Trypsin can be used to collect the adherent cells prior to FACS analysis.

   a. DIC Fluorescent Azide Total DNA
   b. Hydroxyurea
   c. Fluorescence

![Figure: Analysis of newly synthesized DNA in proliferating cells by.](image)

VIII. RELATED PRODUCTS

EZClick™ Global RNA Synthesis Assay Kit (FACS/Microscopy), Red Fluorescence (K718)
EZClick™ Global Phospholipid Synthesis Assay Kit (FACS/Microscopy), Red Fluorescence (K717)
EZCell™ Glutathione Detection Kit (Blue Fluorescence) (K504)