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EZClick™ Global Protein Synthesis Kit (FACS/Microscopy), Red

(Catalog # K715-100; 100 assays; Store at -20 °C) *rev 12/20*

I. Introduction:

Cells generate a complete set of proteins during division. Protein synthesis is a tightly regulated process and many critical controls in gene expression occur at the level of translation to ensure that production of specific cellular proteins is quickly turned on/off under specific conditions (heat shock, starvation, etc.). Protein synthesis is essential for cell growth, proliferation, signaling, differentiation or death. Therefore, the identity and amount of the synthesized proteins are critical parameters in determining the physiological state of the cell. Methods enabling detection and characterization of nascent proteins, or changes in spatial and temporal protein expression/degradation patterns during disease, drug treatments or environmental changes are important tools in assessment of cytotoxicity. BioVision's **EZClick™ Global Protein Synthesis Assay Kit** utilizes a novel and robust chemical method based on an alkyne containing and cell-permeable analog of puromycin, (*O*-propargyl)puromycin (OP-puro). Once inside the cell, OP-puro stops translation by forming covalent conjugates with nascent polypeptide chains. Truncated polypeptides are rapidly turned over by the proteasome and can be detected based on a click reaction with the fluorescent azide. Unlike methionine analogs, OP-puro does not require methionine-free conditions and can be used to label nascent proteins directly in the cell culture. Our kit provides sufficient materials for 100 assays to detect nascent proteins synthesized under various physiological conditions, and Cycloheximide, an inhibitor of protein synthesis that serves as an experimental control.

II. Applications:

- Detection of nascent protein biosynthesis.
- Detection of protein expression or degradation patterns in presence of cytotoxic agents.
- Screening for genotoxic compounds and effectors of protein synthesis.

III. Sample Type:

- Suspension or adherent cell cultures

IV. Kit Contents:

Components	K715-100	Cap Code	Part Number
EZClick™ Wash Buffer (10X)	25 ml	NM	K715-100-1
Fixative Solution	10 ml	WM	K715-100-2
Permeabilization Buffer (10X)	25 ml	NM/Blue	K715-100-3
EZClick™ Protein Label (400X)	25 µl	Clear	K715-100-4
Copper Reagent (100X)	100 µl	Blue	K715-100-5
EZClick™ Fluorescent Azide (100X)	100 µl	Red	K715-100-6
Reducing Agent (20X)	500 µl	Yellow	K715-100-7
EZClick™ Total DNA Stain (1000X)	10 µl	Green/Amber	K715-100-8
Cycloheximide (100X)	10 µl	Green	K715-100-9

V. User Supplied Reagents and Equipment:

- Tissue culture vessels and appropriate culturing media
- PBS, pH 7.4
- Sterile 0.1% Gelatin Solution (optional, only required for suspension cells)
- Fluorescence microscope capable of excitation and emission at 440/490 nm and 540/580 nm respectively

VI. Storage Conditions and Reagent Preparation:

Upon arrival, store the kit at -20° C protected from light. Briefly centrifuge small vials prior to opening. Read the entire protocol before performing the assay.

- **EZClick™ Wash Buffer (10X)** and **Permeabilization Buffer (10X):** Thaw at 37 °C to dissolve completely. Dilute the 10X stocks 1:10 in sterile water, mix well. Store diluted 1X buffer solutions at 4 °C under sterile conditions.
- **Fixative Solution: Aliquot and store at -20 °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 using 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, number of cells per well and other factors may affect the incorporation rate of the Protein Label. Therefore, **optimize the assay for your cell type.** We suggest an initial test of several EZClick™ Protein Label concentrations to find best conditions for your experimental design. Avoid stressing the cells by washes or temperature changes prior to incubation with EZClick™ Protein Label. All steps should be carried out at room temperature (RT) unless otherwise specified. Equilibrate all buffers to RT prior to the experiment.

1. Labeling of control and experimental cells:

Method with drug pre-incubation:

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. Next day, 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 Protein Label or treatment), **Positive Control** (cells incubated with 1X Protein Label only).

b. Replace the media with fresh aliquots containing EZClick™ Protein Label (400X) diluted to 1X final concentration alone, with tested compounds or with Cycloheximide. To use the Cycloheximide, an inhibitor of protein synthesis, dilute at 1:100 in the culture medium. Add Protein Label alone to the Positive Control cells and protein label with Cyclohexamide or test compounds to the experimental cells. Incubate the cells for additional 0.5-2 hours in a 37 °C incubator, or for the period of time as required by your experimental protocol.

c. Terminate the experiment by removing the culture medium and wash the cells once with 100 µl of PBS and discard the supernatant. For immobilized suspension cells: Centrifuge the plate at 300 x g (or the lowest centrifuge setting) for 5 min 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 for aspiration, and perform subsequent aspirations from the same place. Proceed to the Fixation and Permeabilization step.

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 solution and wash the cells once with 200 µl of 1X Wash Buffer, then aspirate the wash. Add 100 µl of 1X Permeabilization Buffer and incubate the cells for 10 min at RT. Remove the Permeabilization Buffer.

b. For suspension cells: Re-suspend the cells in 100 µl of Fixative Solution and incubate for 15 min at RT protected from light. Centrifuge cells at 900 x *g* for 5 min and aspirate the fixative solution. Wash the cells once with 200 µl of 1X Wash Buffer. Centrifuge cells at 900 x *g* for 5 min, discard the supernatant and re-suspend the cells in 100 µl of 1X Permeabilization Buffer. Incubate the cells for 10 min at RT. Centrifuge cells at 900 x *g* for 5 min and remove the Permeabilization Buffer. Proceed to EZClick™ Protein reaction and total DNA staining.

3. EZClick™ Protein reaction and total DNA staining:

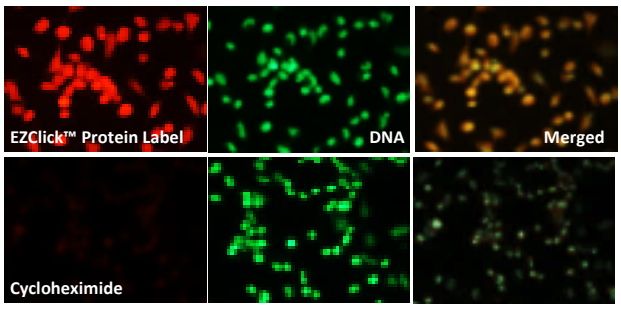
a. Reaction Cocktail: Prepare 1X EZClick™ Reaction Cocktail according to the table below. Volumes should be multiplied by number of Samples and reagents added in the exact order. Use the Reaction Cocktail within 15 min of preparation. Cells should be protected from light during, and following the EZClick™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

	Reaction Cocktail (Amount per Reaction)
PBS	93 µl
Copper Reagent (100X)	1 µl
EZClick™ Fluorescent Azide (100X)	1 µl
Reducing Agent (20X)	5 µl

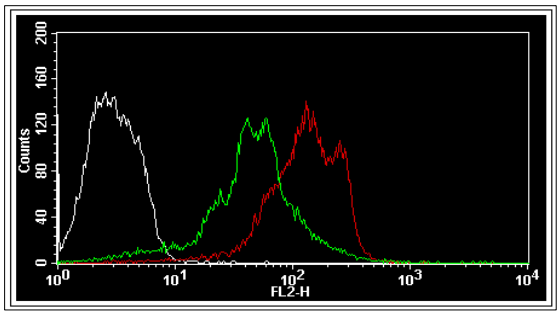
b. EZClick™ Protein Reaction: Add 100 µl of 1X EZClick™ Reaction Cocktail to each sample and incubate the cells for 30 min at RT protected from light. Centrifuge cells at 900 x *g* for 5 min and remove the Reaction Cocktail. Wash cells in 200 µl of Wash Buffer. Centrifuge cells at 900 x *g* for 5 min, aspirate the wash buffer and suspend the cells in 100 µl of PBS. Proceed to DNA staining. If no DNA staining is desired, proceed to Microscopic or FACS analysis. **DNA staining:** Prepare 1X dilution of Total DNA Stain and add 100 µl per well. Incubate the cells for 20 min at RT, or refrigerate at 4°C protected from light. Centrifuge cells at 900 x *g* for 5 min and remove the DNA stain solution; wash the cells once with 200 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 Protein and for green fluorescence by nuclear DNA. **FACS analysis**: Harvest the cells by preferred method and wash with 0.5 ml of ice-cold PBS. Re-suspend the pellets with 100 µl of ice-cold PBS and analyze Samples for red fluorescence generated by *de novo* synthesized protein during click reaction.

A)



B)



Figures: Inhibitory effect of Cycloheximide on nascent polypeptides synthesis. HeLa (10^5 cells/ ml) and Jurkat ($1x10^6$ cells/ml) cells respectively were pre-treated with vehicle or Cycloheximide for 30 min at 37 °C. Subsequently, cells were incubated for additional 30 min with fresh aliquots of media containing either EZClick™ Protein Label or EZClick™ Protein Label and Cycloheximide. Cells were then processed and analyzed by Microscopy and FACS according to the kit protocol. **(A)** Red fluorescence (upper panel) corresponds to *de novo* synthesized polypeptides whereas bottom panel shows the inhibitory effect of Cycloheximide on protein biosynthesis. Nuclear staining in both panels confirms that red signal is a result of Protein Label incorporation. **(B)** FACS analysis of negative control (white), positive control (Protein Label, red) and Cycloheximide-treated (green) cell populations. Signal measured in FL-2 channel clearly shows the inhibitory effect of Cycloheximide on nascent polypeptides synthesis.

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)
EZClick™ EdU Cell Proliferation/DNA Synthesis Kit (FACS/Microscopy), Red Fluorescence (K946)

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