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EZClick™ Global Protein Synthesis Assay Kit

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(Catalog # K467-100; 100 assays; Store at -20°C)

I. Introduction:

Protein synthesis is a tightly regulated process. Many critical controls in gene expression occur at the level of translation to ensure that the production of specific cellular proteins is quickly turned on/off under specific conditions (heat shock, starvation, etc). It is essential in cell growth, proliferation, signaling, differentiation or death. Therefore, the identity and amount of the synthesized proteins are critical in determining the physiological state of the cell. Methods of 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 for assessment of cytotoxicity. **Biovision's EZClick™ Global Protein Synthesis Assay Kit** utilizes a novel chemical method based on alkyne analog of puromycin, *O*-Propargyl-puromycin (OP-puro). OP-puro stops translation by forming covalent conjugates with the 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 (Ex/Em = 494/521 nm). OP-puro does not require methionine-free conditions and can be used to label nascent proteins directly in the cell culture medium. We provide sufficient materials for 100 simple and specific assays to detect nascent proteins synthesized under various physiological conditions in real-time, and in the presence of Cycloheximide, an inhibitor of protein synthesis that serves as a 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 cells

IV. Kit Contents:

Components	K467-100	Cap Code	Part Number
EZClick™ Wash Buffer (10X)	25 ml	NM	K467-100-1
Fixative Solution	10 ml	WM	K467-100-2
Permeabilization Buffer (10X)	25 ml	NM/Blue	K467-100-3
EZClick™ Protein Label (400X)	25 µl	Clear	K467-100-4
Copper Reagent (100X)	100 µl	Blue	K467-100-5
EZClick™ Fluorescent Azide (100X)	100 µl	Green/Amber Vial	K467-100-6
Reducing Agent (20X)	500 µl	Yellow	K467-100-7
EZClick™ Total DNA Stain (1000X)	20 µl	Blue/Amber Vial	K467-100-8
Cycloheximide (100X)	10 µl	Green	K467-100-9

V. User Supplied Reagents and Equipment:

- Tissue culture vessels and appropriate cell media
- 6-, 12-, 24-, or 96-well clear plates should be used only for cell culture. The measurement of fluorescence should be performed in white opaque plates. Alternatively, sterile opaque plates can be used for both, culturing and measurements
- Phosphate Buffered Saline (PBS, pH 7.4)
- Multi-well spectrophotometer and Fluorescence microscope (optional) capable of measuring Ex/Em = 494/521 nm spectra

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 Buffer:** Thaw at 37°C to dissolve completely. Dilute the 10X stocks at 1:10 dilution in sterile water, mix well. Store at 4°C.
- **Fixative Solution: Divide into aliquots 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. Global Protein Synthesis Assay Protocol:

Notes:

This assay was developed with Jurkat (suspension) and HeLa (adherent) cells and can be modified for any cell line. The following protocol has been optimized for a 96-well white opaque plate at 1 X 10^6 cells per well, using fewer cells per well or clear plate will result in decreased signal. We suggest testing growth conditions, cell number per well and several concentrations of the EZClick™ Protein Label, to find the best experimental design for your cell type. The assay volume is 100 µl; adjust volumes accordingly for other plate formats. Avoid stressing 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:

a. Plate suspension or adherent cells at a desired density and allow for overnight recovery before treatment. Ensure that adherent cells are sub-confluent. Include appropriate controls and account for cell loss during the processing. **Negative Control** (cells not exposed to the Protein Label or treatment), **Background Control** (cells treated with EZClick cocktail only), **Positive Control** (cells incubated with 1X Protein Label only).

b. To use Cycloheximide as an inhibitor of protein synthesis, dilute it 1:100 in the culture medium and incubate cells for 30 min at 37°C, remove the media. For **Suspension Cells**: Centrifuge the plate at 500 x *g* (or the lowest centrifuge setting) for 5 min at RT to pellet the cells. Tilt the plate and ***gently*** remove the media with a pipette tip. Avoid excessive centrifugation speeds, which can damage the cells. *Use these centrifugation settings throughout the entire protocol!*

c. Replace the media with fresh aliquots containing 1X EZClick™ Protein Label and tested compound(s), condition(s) or Cycloheximide. Add Protein Label to the **Positive Control** cells. Incubate the cells for additional 0.5-24 hr, or time required by your experimental protocol. **Note:** for drug and EZClick™ Protein Label **co-incubation**, dilute EZClick™ Protein Label directly into the drug or Cycloheximide treated cells, *do not change the media*.

d. Terminate the experiment by removal of the culture media. Harvest the **suspension cells** by centrifugation. **Optional:** detach **adherent cells** (e.g. trypsinize and quench with media), and harvest by centrifugation. Wash the cells once with 100 µl of PBS, discard the supernatant and proceed to the Fixation and Permeabilization.

2. Fixation and Permeabilization:

a. **For adherent and suspension 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 cells once with 100 µl of 1X Wash Buffer, remove wash solution. Add 100 µl of 1X Permeabilization Buffer and incubate the cells for 10 min at RT. Remove the Permeabilization Buffer.Proceed to EZClick™ Protein reaction.

3. EZClick™ Protein reaction:

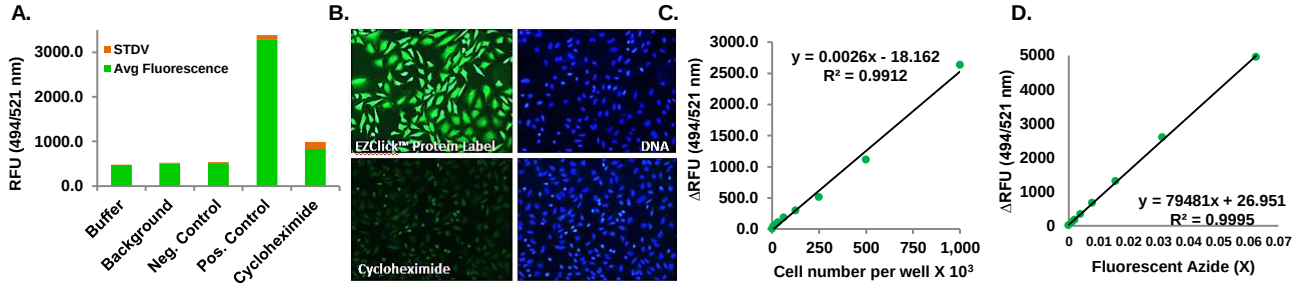
a. **Reaction cocktail:** Prepare 1X EZClick™ reaction cocktail according to the table below. Volumes should be multiplied by number of samples and reagents and added in the exact order. Use the reaction cocktail within 15 minutes of preparation. *Cells should be protected from light during, and following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

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

b. For Negative Control Cells: add 100 µl of 1X PBS. For Background Control Cells, Positive Control Cells and Experimental Cells: add 100 µl of 1X EZClick™ Reaction cocktail to each sample and incubate the cells for 30 min at RT protected from light. Remove the reaction cocktail and wash cells three times in 100 µl of Wash Buffer. Suspend the cells in 100 µl of Wash Buffer.

c. **Detection:** Cells must be analyzed immediately in the plate reader at Ex/Em 494/521 nm in end point mode to determine change in fluorescence of compounds and controls after background subtraction or imaged with fluorescence microscope directly in the plate following DNA staining. **DNA staining:** Prepare 2X dilution of Total DNA Stain and add 100 µl per well. Incubate the cells for 20 minutes at RT, or refrigerate at 4 °C protected from light. Remove the DNA stain and re-suspend the cells in 100 µl of PBS prior to imaging. Analyze samples for green fluorescence generated by *de novo* synthesized protein and for blue fluorescence by nuclear DNA. Note: cells are compatible with all methods of slide preparation including wet or prepared mounting media.

d. **Azide Fluorescence Curve:** To increase the accuracy of your data, an azide fluorescence curve should be prepared from the same cell suspension in parallel to the experimental treatment for each cell line and condition. In a 96-well white opaque plate, prepare a series of dilutions of your cell suspension starting with the same volume and number of cells as in the experimental wells. Dilute the cells by factor of 2. **Optional:** To minimize the error, aliquot at least 3 wells per dilution. Measure fluorescence and calculate average for each dilution, subtract the background value. Plot the Azide Fluorescence Curve to obtain fluorescence per cell number and the detection limit for your assay. Also, standard curve of Fluorescent Azide concentration per well can be prepared in the same manner to obtain the least detectable amount of azide for your experiment.



Figures: Inhibitory effect of Cycloheximide on nascent polypeptides synthesis. Jurkat cells (1X10^6 cells/well) or HeLa cells seeded at 10^5 cells/ ml were pre-treated with vehicle or Cycloheximide for 30 min at 37°C followed by incubation with EZClick™ Protein Label or EZClick™ Protein Label and Cycloheximide for additional 3 h then processed for detection according to the kit protocol. **(A)** Jurkat cells: plate reader analyses of controls and Cycloheximide treatment; Avg fluorescence +/- standard deviation plotted for 3 replicates per condition. **(B)** HeLa cells: upper panel- green fluorescence of *de novo* synthesized peptides; bottom- panel cells treated with 1X Cycloheximide. Nuclear staining confirms that the green signal results from Protein Label incorporation. **(C) Fluorescence Azide Curve of Jurkat cells prepared for this assay.** Detection limit corresponds to about 31,250 of Jurkat cells per well. *Your results may not be identical to these. A new curve must be obtained for each experiment and the cell line*. **(D) Azide Fluorescence Curve in 0-0.1 X range**. *This is reference data and it should not be used to interpret actual results. Your data will depend on the cell type and tested compound.*

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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