Global Protein Synthesis Assay Kit (FACS/Microscopy), Red Fluorescence (#BN00935)

(Catalog # BN00935; 100 assays; Store at -20°C)

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 in 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. Assay Genie's Protein Synthesis Monitoring 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	BN00935	Cap Code	Part Number
Wash Buffer (10X)	25 ml	NM	BN00935-1
Fixative Solution	10 ml	WM	BN00935-2
Permeabilization Buffer (10X)	25 ml	NM/Blue	BN00935-3
Protein Label (400X)	25 µl	Clear	BN00935-4
Copper Reagent (100X)	100 µl	Blue	BN00935-5
Fluorescent Azide (100X)	100 µl	Red	BN00935-6
Reducing Agent (20X)	500 µl	Yellow	BN00935-7
Total DNA Stain (1000X)	10 µl	Green/Amber Vial	BN00935-8
Cycloheximide (100X)	10 µl	Green	BN00935-9

V. User Supplied Reagents and Equipment:

- Tissue culture vessels and appropriate culturing media
- Phosphate Buffered Saline (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 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 Protein Label; therefore optimize the assay for your cell type. We suggest an initial test of several Protein Label concentrations to find best conditions for your experimental design. Avoid stressing the cells by washes or temperature changes prior to incubation with Protein 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; 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. To use the Cycloheximide as an inhibitor of protein synthesis, dilute it 1:100 in the culture medium and incubate the cells for 30 minutes at a 37°C. Remove the drug-containing media. **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. Pellet the* ***suspension cells*** *at 300 x g for 5 min throughout the entire protocol!*

c. Replace the media with fresh aliquots containing Protein Label (400X) diluted to 1X final concentration and tested compound or Cycloheximide. Add Protein Label to the positive control cells. Incubate the cells for additional 0.5 - 2 hours in a 37°C incubator, or for the period of time required by your experimental protocol. **Note**: for drug and Protein Label **co-incubation**, dilute Protein Label directly into the drug or Cycloheximide treated cells, do not change the media.

d. Terminate the experiment by removal of the culture medium and rinse the cells once with 100 µl of PBS, discard the supernatant. Proceed to the Fixation and Permeabilization.

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 Protein reaction and total DNA staining.

3. Protein reaction and total DNA staining:

a. **Reaction cocktail:** Prepare 1X 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 reaction and DNA staining.*

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

b. **Protein Reaction:** Add 100 µl of 1X 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. Remove the wash 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 minutes at RT, or refrigerate at 4 °C protected from light. Remove the DNA 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 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.

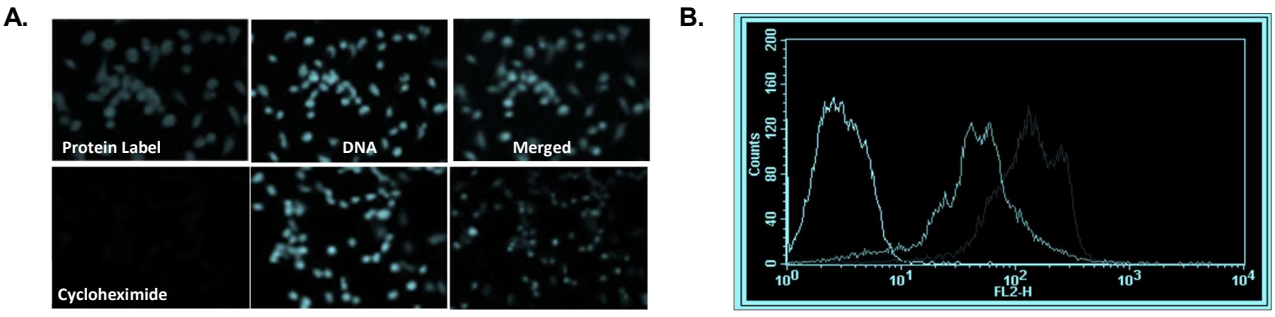

Figure: Inhibitory effect of Cycloheximide on nascent polypeptides synthesis. HeLa (10^5 cells/ ml) and Jurkat ($1X10^6$ cells/ml) cells respectively were pre-treated with vehicle or Cycloheximide for 30 min at 37°C. Subsequently, cells were incubated for additional 30 min with fresh aliquots of media containing either Protein Label or 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.

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