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EZClick™ Stearoylated Protein Assay Kit (FACS/Microscopy), Green

rev 07/20

(Catalog # K453-100; 100 assays; Store at -20°C)

I. Introduction:

Stearoylation is one of the numerous types of post-translational modification in proteins. Stearoylated proteins are involved in the regulation of cellular processes such as apoptosis, growth and metabolism. In this modification, Stearic acid (a saturated fatty acid) is covalently bound to a protein by long-chain-fatty-acid-[acyl-carrier-protein] ligase. In the presence of ATP, this enzyme transfers a Stearate moiety to the target protein forming a carbon-sulfur bond (C=S). Recent studies showed a possible correlation between mitochondrial dysfunction with some neurodegenerative diseases, cancer and aging. Furthermore, it is known that stearoylated Transferrin Receptor (TfR1) inhibits JNK activation, reduces mitofusin ubiquitination, thus restoring mitochondrial function. Studying of stearoylation of proteins can shed a light for disease etiology and prevention. **BioVision offers EZClick™ Stearoylated Protein Assay Kit,** a highly specific, simple and robust method for labeling and detection of Stearoylated proteins. The kit uses Alkynyl Stearic Acid that is fed directly into the cells and gets incorporated into proteins during or post translation. This modification can be followed by click reaction with an azide-containing dye. The assay kit offers a powerful method for imaging localization, trafficking, and dynamics of Stearoylated proteins, or detection by FACS for quantitative studies. Labeled Stearoylated Proteins can be directly detected in 1D or 2D gels using the appropriate excitation sources, or enriched by immunoprecipitation with biotin-azide or antibodies prior to proteomic analysis. We provide sufficient materials for 100 assays in a 96-well plate format.

II. Applications:

- Identification and localization of stearic acid modified proteins.
- Detection and quantification of biosynthesis, dynamics and turnover of stearic acid modified proteins.
- Screening for genotoxic compounds and effectors of protein modifications.

III. Sample Type:

- Suspension or adherent cell cultures

IV. Kit Contents:

Components	K453-100	Cap Code	Part Number
EZClick™ Wash Buffer (10X)	25 ml	NM	K453-100-1
Fixative Solution	10 ml	WM	K453-100-2
Permeabilization Buffer (10X)	25 ml	NM/Blue	K453-100-3
EZClick™ Stearic Acid Label (1000X)	10 µl	White	K453-100-4
Copper Reagent (100X)	100 µl	Blue	K453-100-5
EZClick™ Fluorescent Azide (100X)	100 µl	Green/Amber	K453-100-6
Reducing Agent (20X)	500 µl	Yellow	K453-100-7
EZClick™ Total DNA Stain (1000X)	20 µl	Blue/Amber	K453-100-8

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)
- Flow cytometer equipped with laser capable of excitation at 488 nm wavelength (FL-1)
- Fluorescence microscope capable of excitation and emission at 440/490 nm (Ex =440/Em=490 nm) and UV filter

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:** Thaw at 37°C to dissolve completely. Dilute the 10X stocks 1:10 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. Stearoylated Protein Assay Protocol:

Notes: This assay was developed with HeLa (adherent) and Jurkat (suspension) cells and can be modified for any cell lines. 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 EZClick™ Stearic Acid Label concentrations to find best conditions for your experimental design. Avoid stressing the cells by washes or temperature changes prior to incubation with EZClick™ Stearic Acid 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 <u>drug pre-incubation</u>:**
   a. Obtain cell suspension of desired density and seed directly into tissue culture vessels, or on coverslips for high resolution microscopy. **<u>To immobilize suspension cells for microscopy</u>**: 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*. **<u>Negative Control Cells</u>** (Unstained Cells, cells not exposed to Stearic Acid Label or EZClick™ Fluorescent Azide), **<u>Background Control Cells</u>** (Cells are not exposed to the EZClick™ Stearic Acid Label, are treated with EZClick™ Reaction only), **<u>Positive Control Cells</u>** (Cells are incubated with 1X EZClick™ Stearic Acid Label and EZClick™ Reaction).

b. Next day, for **adherent cells**, remove the media directly. For **suspension cells**, centrifuge cells at 500 x *g* for 5 min, and discard the supernatant. Then replace it with fresh aliquots containing EZClick™ Stearic Acid Label (1000X) diluted to 1X final concentration with culture medium and add into the experimental and positive control cells respectively. *Do not add* the EZClick™ Stearic Acid Label into the **Negative Control Cells**.

c. Add treatments and incubate the cells for additional 1 day or for the period of time as required by your experimental protocol. Do not remove the drug containing media while incubating with 1X EZClick™ Stearic Acid Label to avoid potential reversibility of drug action on label incorporation.

d. To terminate the experiment, **For adherent cells:** Remove the media and rinse the cells once with 100 µl of 1X PBS, discard the supernatant. **For suspension cells**: Centrifuge the cells at 500 x *g* 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 500 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 once 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 supernatant and proceed to EZClick™ Stearic Acid reaction and total DNA staining.

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 the cells at 500 x *g* for 5 minutes, remove the fixative and wash the cells once with 100 µl of 1X Wash Buffer. Centrifuge the cells at 500 x *g* for 5 minutes, discard the supernatant and re-suspend the cells in 100 µl of 1X Permeabilization Buffer. Incubate the cells for 10 min at RT. Centrifuge the cells at 500 x *g* for 5 minutes, remove the supernatant. Proceed to EZClick™ Stearic Acid reaction and total DNA staining.

3. EZClick™ Stearic Acid reaction and total DNA staining:

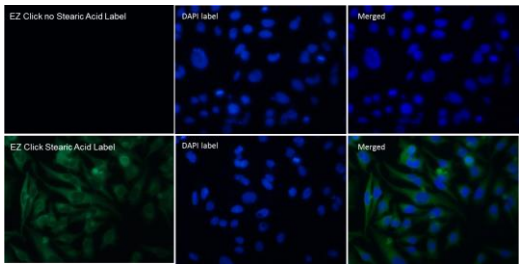
a. Reaction cocktail: Prepare 1X EZClick™ reaction cocktail according to the table below. Volumes should be multiplied by number of samples and reagents added in the exact order. Use the reaction cocktail within 15 minutes of preparation. *Cells should be protected from light during, and following the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

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

b. EZClick™ Stearic Acid Reaction: 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 1X Wash Buffer. Remove the 1X wash and suspend the cells in 100 µl of 1X 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 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 green fluorescence generated by EZClick™ labeled Stearic Acid and for blue fluorescence by nuclear DNA. **FACS analysis**: Harvest cells by preferred method and wash with 0.5 ml of ice-cold PBS. Re-suspend pellets with 100 µl of ice-cold PBS and analyze samples for green fluorescence generated by EZClick™ Stearic Acid addition during EZClick™ reaction.

A.



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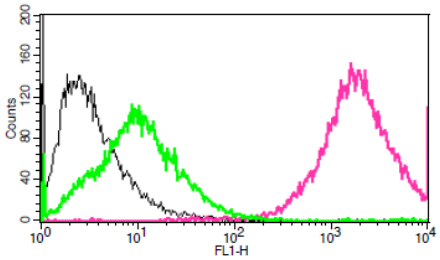

Figures: Analysis of metabolic labeling of Stearic Acid on proliferating cells. HeLa (10^5 cells/ml) and Jurkat ($1X10^6$ cells/ml) cells respectively were incubated overnight with fresh aliquots of media containing EZClick™ Stearic Acid Label. Cells were then processed and analyzed by Microscopy and FACS according to the kit protocol. **(A) HeLa cells**: Upper panel corresponds to the Azide only Background fluorescence. The lower panel shows that the green fluorescence corresponds to the EZclick™ Stearic Acid labeling. Nuclear staining in both panels confirms that green signal is a result of Stearic Acid Label incorporation. **(B) Jurkat cells:** FACS analysis of negative control (Black), background (EZClick only, Green), positive control (Stearic Acid Label and EZClick, Pink). Signal measured in FL-1 channel clearly shows the Stearic Acid Labeling of Protein.

VIII. Related EZClick™ Products:

EZClick™ Palmitoylated Protein Assay Kit (FACS/Microscopy), Green Fluorescence (K497)
EZClick™ Palmitoylated Protein Assay Kit (FACS/Microscopy), Red Fluorescence (K416)
Global Phospholipid Assay Kit (K717)

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