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# EZClick™ Palmitoylated Protein Assay Kit (FACS/Microscopy), Red Fluorescence

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**(Catalog # K416-100; 100 assays; Store at -20°C)**

## I. Introduction:

Palmitoylation occurs when fatty acids, such as Palmitic Acid are covalently attached to the side chains of cysteine (and less frequently to serine, threonine side chains) in proteins. This type of post-translational modification greatly affects cellular trafficking, compartmentalization, and membrane tethering. Palmityltransferase (PAT), the enzyme responsible for this modification, transfers a palmitate moiety from palmitoyl-CoA to the thiol group of cysteine in the target protein. Compared to myristoylation, palmitoylation is reversible and the reverse reaction is catalyzed by thioesterases. The Palmitoylation/Depalmitoylation cycle plays an important role when modified protein shuttles between cellular compartments. PAT mutations are associated with many neurological diseases and cancer progression. BioVision offers EZClick™ Palmitoylated Protein Assay Kit, a highly specific, simple and robust method for labeling and detection of palmitoylated proteins. The kit uses a modified Palmitic Acid that is fed directly into the cells and gets incorporated into proteins during or post translation. This post translational 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 Palmitoylated proteins, or detection by FACS for quantitative studies. Labeled Palmitoylated 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 Palmitic acid modified proteins
- Detection and quantification of biosynthesis, dynamics and turnover of palmitoylated proteins
- Screening for genotoxic compounds and effectors of protein modifications

## III. Sample Type:

- Suspension or adherent cell cultures

## IV. Kit Contents:

| Components | K416-100 | Cap Code | Part Number |
|---|---|---|---|
| EZClick™ Wash Buffer (10X) | 25 ml | NM | K416-100-1 |
| Fixative Solution | 10 ml | WM | K416-100-2 |
| Permeabilization Buffer (10X) | 25 ml | Blue NM | K416-100-3 |
| EZClick™ Palmitic Acid Label (1000X) | 10 µl | White | K416-100-4 |
| Copper Reagent (100X) | 100 µl | Blue | K416-100-5 |
| EZClick™ Fluorescent Azide (100X) | 100 µl | Red | K416-100-6 |
| Reducing Agent (20X) | 500 µl | Yellow | K416-100-7 |
| EZClick™ Total DNA Stain (1000X) | 20 µl | Blue/Amber | K416-100-8 |

## 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 540/580 nm wavelength (FL-2)
- Fluorescence microscope capable of excitation and emission at 440/490 nm and 540/580 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:** 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. Palmitoylated Protein Assay Protocol:

**Notes:**

This assay was developed using 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™ Palmitic Acid Label concentrations to find the best condition for your experimental design. Avoid stressing the cells by washes or temperature changes prior to incubation with EZClick™ Palmitic 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. D*o not add treatment to the positive and negative control cells*. **<u>Negative Control Cells</u>** (Unstained Cells, cells not

exposed to Palmitic Acid Label or EZClick™ Fluorescent Azide); **Background Control Cells** (Cells are not exposed to the EZClick™ Palmitic Acid Label, EZClick™ Reaction only), **Positive Control Cells** (Cells are incubated with 1X EZClick™ Palmitic Acid Label and EZClick™ Reaction).

**b.** Next day, for **adherent cells**, remove the media directly. For **suspension cells**, centrifuge cells at 300 x *g* for 5 min, and discard the supernatant. Then replace it with fresh aliquots containing EZClick™ Palmitic 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™ Palmitic Acid Label into the **Negative Control Cells**.

**c.** Add treatments and incubate the cells for additional 1 day or for the period of time required by your experimental protocol. Do not remove the drug containing media while incubating with 1X EZClick™ Palmitic 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 300 x *g* for 5 minutes to deposit the cells onto the surface. Tilt the plate and ***gently*** remove the media with a pipette tip. Avoid excessive centrifugation speed and repeated cycles, which could induce cell damage. *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!*

**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 replace it with a 20 µl of 1X 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 the cells at 300 x *g* for 5 minutes, remove the fixative and wash the cells once with 100 µl of 1X Wash Buffer. Centrifuge the cells at 300 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 300 x *g* for 5 minutes. Remove the supernatant and replace it with a 20 µl of Permeabilization Buffer. Proceed to EZClick™ Palmitic Acid reaction and total DNA staining.

**3. EZClick™ Palmitic Acid reaction and total DNA staining:**

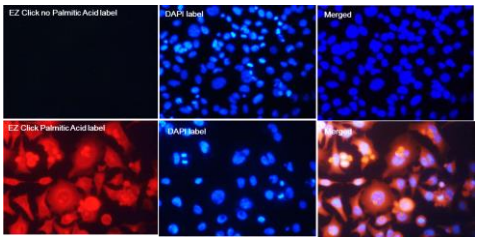
**a. Reaction Cocktail:** Prepare 1X EZClick™ Reaction Cocktail according to the table below. Volumes should be multiplied by number of Samples and reagents added in the exact order. Use the Reaction Cocktail within 15 minutes of preparation. *Cells should be protected from light during, and following 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™ Palmitic 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 red fluorescence generated by EZClick™ labeled Palmitic 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 red fluorescence generated by EZClick™ Palmitic Acid addition during EZClick™ reaction.

**A.**




**B.**

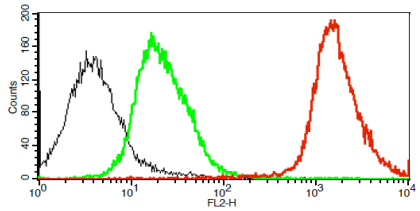



**Figure: Analysis of metabolic labeling of Palmitic Acid on proliferating cells.** HeLa ($10^5$ cells/ ml) and Jurkat ($1X10^6$ cells/ml) cells respectively were incubated overnight with fresh aliquots of media containing EZClick™ Palmitic Acid Label. Cells were then processed and analyzed by Microscopy 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 red fluorescence corresponds to the EZclick™ Palmitic Acid labeling. Nuclear staining in both panels confirms that green signal is a result of Palmitic Acid Label incorporation. **(B) Jurkat cells:** FACS analysis of Negative Control (Black), Background (EZClick only, Green), Positive Control (Palmitic Acid Label and EZClick, Red). Signal measured in FL-2 channel clearly shows the Palmitic Acid Labeling of Protein.

## VIII. RELATED EZClick™ PRODUCTS:

EZClick™ Myristoylated Protein Assay Kit (FACS/Microscopy), Green Fluorescence (K497)
EZClick™ Myristoylated Protein Assay Kit (FACS/Microscopy), Red Fluorescence (K177)
Global Phospholipid Assay Kit (K717)

***FOR RESEARCH USE ONLY! Not to be used on humans.***