EZClick™ Total Phospholipid Assay Kit (Cell-Based)

rev 07/20

(Catalog # K537-100; 100 assays; Store at -20°C)

I. Introduction:

Phospholipids are major component of the bilayers of all plasma membranes. A single phospholipid molecule consists of a phosphate group on one ("head"), and two side-by-side chains of fatty acids that make up the "tails". The phosphate head groups can be modified with organic molecules such as Choline (Cho). Cho-containing phospholipids (Phosphatidylcholines; PC) are critical for structural membrane integrity, cellular metabolism and signaling either as individual molecules or precursors of secondary messengers. Changes in global synthesis of Cho-containing phospholipids are an essential parameter in analysis of cellular response to both, physiological and pathological conditions, environmental stress, or drug treatment. To date, phospholipids biochemistry, cell biology and metabolism remain obscure, due to limited methods for their direct cellular visualization. **BioVision's EZClick™ Global Phospholipid Synthesis Assay Kit** offers a simple and robust method to label and visualize newly synthesized phospholipids *in vivo*. Based on the metabolic incorporation of the choline analogs directly into their structure, modified phospholipid molecules can be detected with high sensitivity and spatial resolution by click chemistry with azide-containing dyes (Ex/Em= 494/521 nm). This kit enables quantitative analyses of global biosynthesis/turnover of Cho-containing phospholipids in cells. Cells show strong incorporation of Cho analogs into all classes of phospholipids that can be assayed by microplate reader and fluorescence microscope. The kit provides sufficient materials for 100 assays.

II. Applications:

- Detection and quantification of biosynthesis, subcellular localization and turnover of phospholipids
- Evaluating effects of anti-cancer drugs and genotoxic agents on phospholipids
- Screening for genotoxic compounds and effectors of phospholipid biosynthesis in proliferating cells

III. Sample Type:

- Suspension or adherent cell cultures

IV. Kit Contents:

Components	K537-100	Cap Code	Part Number
EZClick™ Wash Buffer (10X)	25 ml	NM	K537-100-1
Fixative Solution	10 ml	WM	K537-100-2
Permeabilization Buffer (10X)	25 ml	NM/Blue	K537-100-3
EZClick™ Phospholipid Label (1000X)	10 µl	Clear	K537-100-4
Copper Reagent (100X)	100 µl	Blue	K537-100-5
EZClick™ Fluorescent Azide (100X)	100 µl	Green/Amber Vial	K537-100-6
Reducing Agent (20X)	500 µl	Yellow	K537-100-7
EZClick™ Total DNA Stain (1000X)	20 µl	Blue/Amber Vial	K537-100-8

V. User Supplied Reagents and Equipment:

- Tissue culture vessels and appropriate culturing media
- A 6-, 12-, 24-, or 96-well clear plates should be used only for cell culturing. The measurement of fluorescence should be performed in black 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 (optionally) 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 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. Total Phospholipid 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 opaque plate at $1X10^6$ 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™ Phospholipid 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 the cells by washes or temperature changes prior to incubation with EZClick™ Phospholipid 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 Phospholipid Label or treatment), **Background control** (cells treated with EZClick cocktail only), **Positive control** (cells incubated with 1X Phospholipid Label only).

b. Next day, remove the media and treat the cells with appropriate effectors according to your protocol; do not add treatment to the positive and negative control cells. For **suspension cells**: Centrifuge the plate at 500 x g (or the lowest centrifuge setting) for 5 minutes 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. Dilute EZClick™ Phospholipid Label (1000X) to 1X final concentration with culture medium and add into the **Experimental** and **Positive control** cells respectively. Incubate the cells for additional 24 hours, or time required by your experimental protocol in a 37°C incubator. Do not add the EZClick™ Phospholipid Label into the **Negative control** cells. Do not remove the drug-containing media during incubation with 1X Phospholipid Label to avoid potential reversibility of drug action on label incorporation. Harvest the

suspension cells by centrifugation. **Optionally**, 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 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 Permeabilization Buffer. Proceed to EZClick™ Phospholipid reaction.

3. EZClick™ Phospholipid reaction:

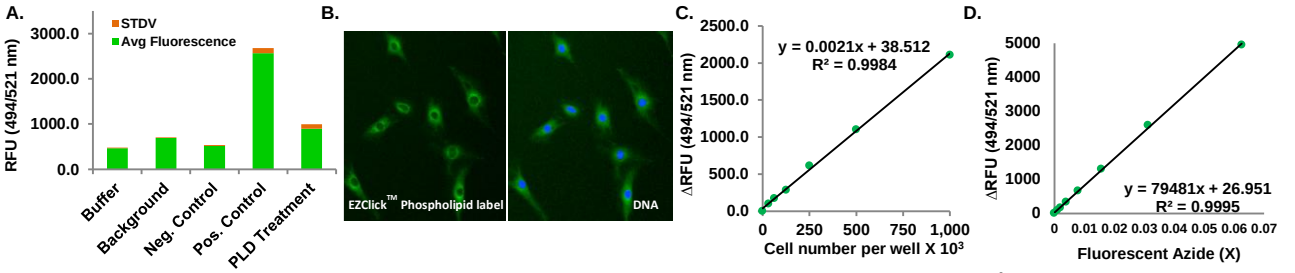
a. **Reaction cocktail:** Prepare 1X EZClick™ reaction cocktail according to the table below. Volumes should be multiplied by number of samples and reagents and added in the exact order. Use the reaction cocktail within 15 minutes of preparation. *Cells should be protected from light during, and following 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 µ 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 phospholipid and for blue fluorescence by nuclear DNA. Note: cells are compatible 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 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: Analysis of metabolic labeling of phospholipids in proliferating cells. Jurkat cells (1X10^6 cells/well) or BALB/3T3 cells seeded at 10^5 cells/ ml were pre-treated with vehicle or cultured in presence of 1X EZClick™ Phospholipid Label for 24 hours at 37°C prior to 1 hour treatment with Phospholipase D and then processed for detection of according to the kit protocol. **(A)** Jurkat cells: plate reader analyses of controls and PLD treatment; Avg fluorescence +/- standard deviation plotted for 3 replicates per condition. **(B)** BALB/3T3 cells: left panel- green fluorescence of *de novo* synthesized phospholipids; right panel-nuclear staining and phospholipid images merged. **(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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