Global Phospholipid Synthesis Assay Kit (FACS/Microscopy), Red Fluorescence (#BN00937)

(Catalog # BN00937; 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 end, called the "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. Assay Genie's 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. This kit enables analyses of global biosynthesis, subcellular localization and turnover of Cho-containing phospholipids in cells. Cells show strong incorporation of Cho analogs into all classes of phospholipids that can be assayed by fluorescence microscopy, or quantified by FACS. We provide sufficient materials for 100 assays.

II. Applications:

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

III. Sample Type:

- Suspension or adherent cell cultures

IV. Kit Contents:

Components	BN00937	Cap Code	Part Number
Wash Buffer (10X)	25 ml	NM	BN00937-1
Fixative Solution	10 ml	WM	BN00937-2
Permeabilization Buffer (10X)	25 ml	NM/Blue	BN00937-3
Phospholipid Label (1000X)	10 µl	Clear	BN00937-4
Copper Reagent (100X)	100 µl	Blue	BN00937-5
Fluorescent Azide (100X)	100 µl	Red	BN00937-6
Reducing Agent (20X)	500 µl	Yellow	BN00937-7
Total DNA Stain (1000X)	10 µl	Green	BN00937-8

V. User Supplied Reagents and Equipment:

- Tissue culture vessels and appropriate culturing media; flow cytometry vessels
- Phosphate Buffered Saline (PBS, pH 7.4)
- Sterile 0.1% Gelatin Solution (optional, only required for adhering suspension cells to the surface)
- Flow cytometer equipped with laser capable of excitation at 488 and 530/590 nm emission filters respectively
- 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 BALB/3T3 (adherent) and Jurkat (suspension) cells and can be modified for any cell line. The protocol below refers to a 96-well tissue culture plate format and assay volume is 100 µl; adjust volumes accordingly for other plate formats. Growth conditions, cell number per well and other factors may affect the incorporation rate of the Phospholipid Label; therefore optimize the assay for your cell type. We suggest an initial test of several Phospholipid Label concentrations to find best conditions for tested cell type and experimental design. Avoid stressing the cells by washes or temperature changes prior to incubation with 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. 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 into each well of a tissue culture plate, 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.

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. **Negative control** -cells not exposed to the Phospholipid Label or treatment), **positive control** (cells incubated with 1X Phospholipid Label only.

c. Dilute Phospholipid Label (1000X) to 1X final concentration with culture medium and add into the experimental and positive control cells respectively. *Do not add the* 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. Incubate the cells for additional 24 hours in a 37°C incubator, or for the period of time required by your experimental protocol.

d. Terminate the experiment by removal of the culture medium and rinsing cells once with 100 µl of PBS, discard the supernatant. **For immobilized suspension cells**: Centrifuge the plate at 300 x g (or the lowest centrifuge setting) for 5 minutes to gently 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*. Proceed to the Fixation and Permeabilization. *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 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 reaction and total DNA staining.

3. Phospholipid 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. **Reaction:** Add 100 µl of 1X Reaction cocktail to each sample and incubate the cells for 30 min at room temperature 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 room temperature, or refrigerate at 4 °C protected from light. Remove the 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 and FACS analysis**: Analyze samples for red fluorescence generated by labeled phospholipid and green by total DNA respectively. **FACS analysis:** Harvest the cells by preferred method and wash with 0.5 ml of ice-cold PBS. Re-suspend the pellets in 100 µl of ice-cold PBS. Transfer the cell suspension into flow cytometry vessels. Analyze samples in FL-2 channel for signal generated by phospholipid during click reaction. **Note:** Trypsin can be used to collect the adherent cells prior to FACS analysis.

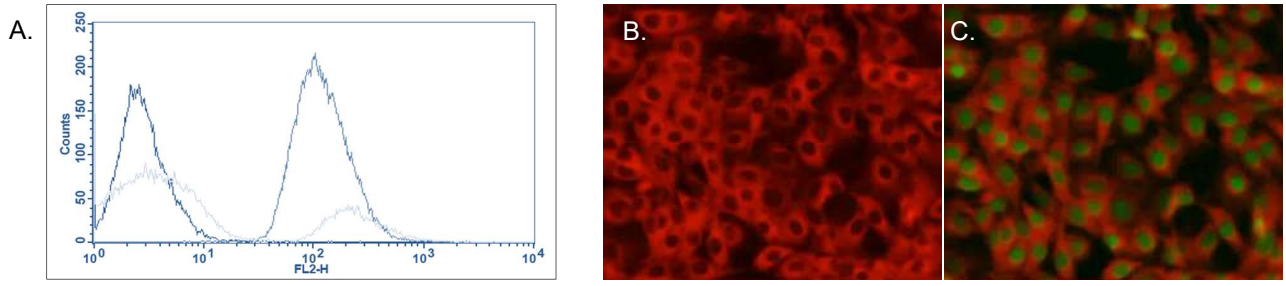

Figure: Analysis of metabolic labeling of phospholipids in proliferating cells. **(A)** Jurkat cells (1X10^6 cells/ml) were pre-treated with vehicle **(black line)** or cultured in presence of 1X Phospholipid Label **(red line)** for 24 hours at 37°C prior to 1 hour incubation with Phospholipase D **(blue line)**. Modified phospholipid molecules were detected according to the kit protocol and red fluorescence was analyzed by FACS in FL-2 channel. Decrease in signal caused by hydrolysis of Cho-containing head groups via Phospholipase D activity confirms that red fluorescence is the result of Phospholipid Label incorporation. **(B)** BALB/3T3 cells (10^5 cells/ ml) cultured in presence of 1X Phospholipid Label for 24 hours at 37°C and processed according to kit protocol. Choline-containing phospholipids were detected by Fluorescence Microscope. according to kit protocol. Total DNA staining **(C)** confirms that red fluorescence is the result of Phospholipid Label incorporation.

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