EZClick™ *O*-GalNAc Modified Glycoprotein Assay Kit (FACS/Microscopy, Green)

rev 06/20

(Catalog # K560-100; 100 assays; Store at -20°C)

I. Introduction:

Glycans are vital components of glycoproteins, glycolipids, and proteoglycans in all domains of life. Glycoproteins are grouped by the type of carbohydrate and amino acid linkage site. *N*-linked glycosylation is a modification of asparagine, whereas *O*-linked glycosylation occurs through the hydroxyl group of serine and threonine residues. Glycosylation occurs co- or post-translationally on >50% of eukaryotic proteins resulting in membrane-associated, intracellular, or secreted glycoproteins that are crucial in cellular processes, protein bioactivity and metabolic turnover. Attachment of *O*-linked mucin-type glycans is a common post-translational modification initiated by the addition of *N*-acetyl-galactosamine (GalNAc) to a Ser or Thr residue of newly assembled protein. Resulting GalNAc-(Ser/Thr) structure (Tn-antigen) can be further modified by the addition of either a sialic acid residue (sialyl-Tn), GlcNAc to form GlcNAc-GalNAc (core 3), or a galactose residue to form the GalGalNAc (core 1) structures respectively. The core 3 and 1structures are then further extended to form long and branched complex *O*-glycans. The core 1 (TF or T antigen) acts like oncofetal antigen, overexpressed in cancerous and precancerous conditions due to the Golgi apparatus disorder. GalNAc linked to the first mannose of glycosylphosphatidylinositol (GPI) core has been previously reported to be heterogeneously present on mammalian GPI-anchored proteins. Thus **BioVision offers EZClick™ O-GalNAc Modified Glycoprotein Assay Kit**, a highly specific, simple and robust method for labeling and detection of O-GalNAc-glycosylated proteins within cells. We use a modified galactosamine precursor that is fed directly into the cells, processed by the GalNAc salvage pathway to form the intermediate uridine diphospho (UDP)-GalNAz, which is recognized by GalNAc transferases in the Golgi and incorporated into the protein. Followed by click reaction with alkyne-containing dye, this system offers a powerful method for imaging the localization, trafficking, and dynamics of glycans, or detection by FACS for quantitative studies. Labeled Glycoproteins can be directly detected in 1D or 2D gels using the appropriate excitation sources, or enriched by immunoprecipitation with biotin-alkyne or antibodies prior to proteomic analysis. We provide sufficient materials for 100 assays in a 96-well plate format.

II. Applications:

- Identification, characterization and profiling of *O*-GalNAc-glycosylated proteins and GalNAc-containing GPI-anchored proteins
- Imaging the localization, trafficking, and dynamics of mucin-type *O*-linked glycans
- Detection and quantification of biosynthesis, subcellular localization and turnover of modified glycans
- Screening for genotoxic compounds and effectors of modified glycans in proliferating cells
- Evaluating effects of anti-cancer drugs and genotoxic agents on modified glycans

III. Sample Type:

- Suspension or adherent cell cultures

IV. Kit Contents:

Components	K560-100	Cap Code	Part Number
EZClick™ Wash Buffer (10X)	25 ml	NM	K560-100-1
Fixative Solution	10 ml	WM	K560-100-2
Permeabilization Buffer (10X)	25 ml	NM/Blue	K560-100-3
EZClick™ GalAz Label (1000X)	10 µl	Clear	K560-100-4
Copper Reagent (100X)	100 µl	Blue	K560-100-5
EZClick™ Fluorescent Alkyne (100X)	100 µl	Green	K560-100-6
Reducing Agent (20X)	500 µl	Yellow	K560-100-7
EZClick™ Total DNA Stain (1000X)	20 µl	Amber/Blue	K560-100-8

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 Buffer:** 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. 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 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 GalAz Label; therefore optimize the assay for your cell type. We suggest an initial test of several GalAz Label concentrations to find best conditions for your experimental design. Avoid stressing the cells by washes or temperature changes prior to incubation with GalAz 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 with GalAz Label:

a. Seed the cell suspension of desired density 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 replace it with fresh aliquots containing 1X EZClick™ GalAz Label. Include appropriate controls. **Negative Control** -cells not exposed to the 1X GalAz Label or treatment, **Positive Control** -cells incubated with 1X GalAz Label only. *Do not add the* EZClick™ GalAz Label *into the* ***Negative Control*** *cells*.

c. Add treatments and incubate the cells for additional 1-3 days in a 37°C incubator, or for the period of time required by your experimental protocol. For analysis of trafficking and dynamics of cellular glycans take samples during incubation. Do not remove the drug-containing media while incubating with 1X GalAz Label to avoid potential reversibility of drug action on label incorporation.

d. Terminate the experiment, remove the media and rinse the cells once with 100 µl of PBS, discard the supernatant. Always pellet the **suspension cells** at 300 x g for 5 min throughout the entire protocol. **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.

2. Fixation and Permeabilization:

a. **For adherent cells:** Add 100 µl of Fixative Solution per well and incubate the cells for 15 min at RT protected from light. Remove the fixative and wash the cells once with 200 µl of 1X Wash Buffer. Remove the wash and add 100 µl of 1X Permeabilization Buffer per well, incubate the cells for 10 min at RT. Remove the Permeabilization Buffer and proceed to EZClick™ GalAz 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 cells at 900 x g for 5 minutes and remove the fixative solution. Wash the cells once with 200 µl of 1X Wash Buffer. Centrifuge cells at 900 x g for 5 minutes and discard the supernatant and re-suspend the cells in 100 µl of 1X Permeabilization Buffer. Incubate the cells for 10 min at RT. Centrifuge cells at 900 x g for 5 minutes and remove the Permeabilization Buffer. Proceed to EZClick™ GalAz reaction and total DNA staining.

3. EZClick™ GalAz reaction and total DNA staining:

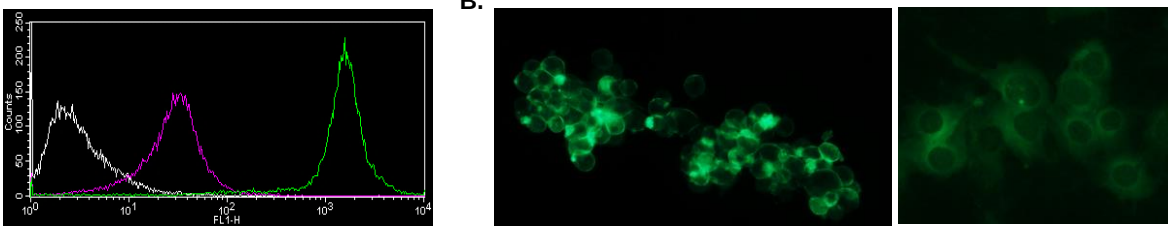
a. Reaction Cocktail: Prepare 1X EZClick™ reaction cocktail according to the table below. Volumes should be multiplied by number of samples and reagents added in the exact order. Use the reaction cocktail within 15 minutes of preparation. *Cells should be protected from light during, and following the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

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

b. EZClick™ Reaction: Add 100 µl of 1X EZClick™ Reaction cocktail to each sample and incubate the cells for 30 min at room temperature protected from light. Centrifuge cells at 900 x g for 5 minutes and remove the reaction cocktail and wash cells in 100 µl of Wash Buffer. Centrifuge cells at 900 x g for 5 minutes and remove the wash solution. Do 2 times wash step 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 EZClick™ 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. Centrifuge cells at 900 x g for 5 min and remove the stain solution and replace with 100 µl of PBS.

4. Fluorescence Microscope analysis: Examine labeled glycoproteins using FITC filter and UV laser for total DNA staining.
FACS analysis: Harvest the cells and wash with 500 µl of PBS. Centrifuge cells at 900 x g for 5 minutes and re-suspend the pellets in 100 µl of ice-cold PBS and transfer the cell suspension into flow cytometry vessels. Analyze samples in FL-1 channel signal generated by labeled glycoproteins.

A. **B.**



Figures: Analysis of metabolic labeling of GalAz labeled glycans in proliferating cells. **(A)** Jurkat cells (1X10^6 cells/ml) were cultured in presence of 1X EZClick™ GalAz Label for 24 hours at 37°C. Modified glycoproteins were detected according to the kit protocol and green fluorescence was analyzed by FACS (FL-1 channel). Negative control **(white line)**, Background control (**purple line**), fluorescence corresponding to intracellular O-GalNAc-glycosylated proteins **(green line)**. **(B)** Fluorescence Microscope images of cell surface (left panel) and subcellular localization (right panel) of O-GalNAc-glycosylated proteins in fixed HeLa cells.

VIII. Related Products:

EZClick™ EdU Cell Proliferation/DNA Synthesis Kit (FACS/Microscopy), Red Fluorescence (K946)
EZClick™ Global RNA Synthesis Assay Kit (FACS/Microscopy), Red Fluorescence (K718)
EZClick™ Global Protein Synthesis Assay Kit (FACS/Microscopy), Red Fluorescence (K715)
EZClick™ Global Phospholipid Synthesis Assay Kit (FACS/Microscopy), Red Fluorescence (K717)
EZClickTM Sialic Acid (ManAz) Modified Glycoprotein Assay Kit (FACS/Microscopy, Green Fluorescence) (K441)
EZClickTM O-GlcNAc Modified Glycoprotein Assay Kit (FACS/Microscopy, Green Fluorescence) (K714)

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