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EZClick™ O-GlcNAc Modified Glycoprotein Assay Kit (FACS/Microscopy, Red)

06/21

(Catalog # K2092-100; 100 assays; Store at -20 °C)

I. Introduction:

Glycans are the vital components of glycoproteins, glycolipids, and proteoglycans. Glycoproteins are grouped by the type of carbohydrate and the amino acid linkage site. N-linked glycosylation is a modification of asparagine, whereas O-linked glycosylation occurs on the hydroxyl group of serine and threonine residues. Glycosylation occurs co- or post-translationally on more than 50% of eukaryotic proteins resulting in membrane-assisted, intracellular, or secreted glycoproteins that are crucial in cellular processes, protein bioactivity and metabolic turnover. Modification by O-linked-N-acetyl glucosamine (O-GlcNAc) has rapidly emerged as a major cellular signaling mechanism with number of modified targets similar to protein phosphorylation. O-GlcAc glycosylation is implicated in normal brain functions, etiology of neurodegeneration, type II diabetes, pathways involved in morphogenesis, and virulence factors of microbes in plant host cells. Since glycoproteins are not directly encoded in the genome, methods of characterization and analyses of glycoproteins are of great interest. **BioVision's EZClick™ O-GlcNAc Modified Glycoprotein Assay Kit** offers a highly specific, simple and robust method for labeling and detection of O-GlcNAc modified glycoproteins. In this assay, a modified glucosamine precursor is fed directly into the cells, which is processed by the hexosamine pathway and is incorporated into proteins. This is followed by the click reaction with an alkyne-containing dye. This system offers a powerful method for imaging the localization, trafficking, 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 and localization of O-GlcNAc-glycosylated proteins within cells.
- Imaging the localization, trafficking, and dynamics of modified glycans.
- Detection and quantification of biosynthesis, dynamics 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 Types:

- Suspension or adherent cell cultures

IV. Kit Contents:

Components	K2092-100	Cap Code	Part Number
EZClick™ Wash Buffer (10X)	25 ml	NM	K2092-100-1
Fixative Solution	10 ml	WM	K2092-100-2
Permeabilization Buffer (10X)	25 ml	Blue/NM	K2092-100-3
EZClick™ GlcAz Label (1000X)	10 µl	Clear	K2092-100-4
Copper Reagent (100X)	100 µl	Blue	K2092-100-5
EZClick™ Fluorescent Alkyne (100X)	100 µl	Red	K2092-100-6
Reducing Agent (20X)	500 µl	Yellow	K2092-100-7
EZClick™ Total DNA Stain (1000X)	20 µl	Amber/Blue	K2092-100-8

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 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 and 540/580 nm.

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.

- **EZClick™ Wash Buffer (10X) and Permeabilization Buffer (10X): Thaw at 37 °C to dissolve completely.** Dilute the 10X stocks at 1:10 dilution in sterile water and mix well. Store at 4 °C.
- **Fixative Solution:** Ready to use**. 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. EZClick™ O-GlcNAc Modified Glycoprotein 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 with an assay volume of 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 GlcAz Label. Therefore optimize the assay depending on your cell type. We suggest an initial test of several EZClick™ GlcAz Label concentrations for your experimental design. Avoid stressing the cells by washes or temperature changes prior to incubation with EZClick™ GlcAz 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 EZClick™ GlcAz Label:**
   - **a.** Seed the cell suspension of desired density directly into the tissue culture vessels, or on coverslips for high resolution microscopy. To immobilize the 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 hr. Gently, remove the gelatin solution and seed the 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™ GlcAz Label. Include the appropriate controls: **Negative Control:** Unstained cells. i.e. cells not exposed to EZClick™ GlcAz Label or any treatments. **Background Control:** Cells are not exposed to EZClick™ GlcAzLabel and contains only EZClick™ Reaction. **Positive Control:** Cells are incubated with 1X EZClick™ GlcAz Label only.
   - **c.** Add the treatment and incubate the cells for an additional 1-3 days in a 37 °C incubator, or for the period of time as 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 GlcAz 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 immobilized suspension cells**: Centrifuge the plate at 500 x g (or the lowest centrifuge setting) for 5 min 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 position in the well that is used for aspiration and perform subsequent aspirations from the same location*. Proceed to the Fixation and Permeabilization step.

2. **Fixation and Permeabilization:**

   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 twice with 100 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™ reaction and total DNA staining.

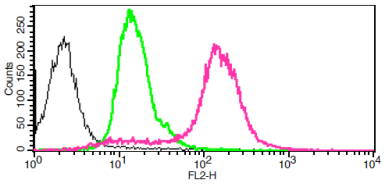
3. **EZClick™ GlcAz Reaction and Total DNA Staining:**
   - **a. EZClick™ Reaction Cocktail:** Prepare 1X EZClick™ Reaction Cocktail according to the table below. Volumes should be multiplied by number of samples and reagents and added in the order listed below. Use the EZClick™ Reaction Cocktail within 15 min of preparation. *Cells should be protected from light during, and after 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:** **For Negative Control:** Add 100 µl of 1X PBS. **For Background Control, 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 final 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 EZClick™ Total DNA Stain and add 100 µl per well. Incubate the cells for 20 min at RT, or refrigerate at 4 °C, protected from light. Remove the DNA staining 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 Analysis:** Examine the samples for red fluorescence generated by EZClick™ labeled GlcAz and blue fluorescence for nuclear DNA. **FACS Analysis:** Harvest the cells by any preferred method and wash with 0.5 ml of ice-cold PBS. Re-suspend the pellets in 100 µl of ice-cold PBS and analyze samples for red fluorescence generated by EZClick™ GlcAz addition during EZClick™ reaction.

A.



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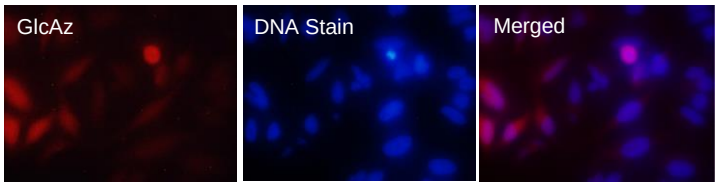

Figures: Analysis of metabolic labeling of GlcAz labeled glycans in proliferating cells. (A) Jurkat cells ($1X10^6$ cells/ml) were incubated in presence of 1X EZClick™ GlcAz for 24 hr at 37 °C. Modified glycoproteins were detected according to the kit protocol and red fluorescence was analyzed by FACS (FL-2 channel): Negative Control (black), Background Control (green) and fluorescence corresponding to intracellular O-GlcNAc-glycosylated proteins (Pink). **(B) HeLa cells**: Fluorescence Microscope analysis of intracellular O-GlcNAc-glycosylated proteins in HeLa cells (40X magnification). The red fluorescence corresponds to the GlcAz labeling. The blue fluorescence corresponds to the DNA staining. The merged image confirms the presence intracellular O-GlcNAc modified glycoproteins.

VIII. Related EZClick™ Products:

EZClick™ Sialic Acid (ManAz) Modified Glycoprotein Assay Kit (FACS/Microscopy), Green Fluorescence (K441)
EZClick™ O-GlcAc Modified Glycoprotein Assay Kit (FACS/Microscopy), Green Fluorescence (K714)
EZClick™ O-GalAc Modified Glycoprotein Assay Kit (FACS/Microscopy), Green Fluorescence (K560)

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