Glycoprotein labeling and detection: Novel "click" chemistry based applications for gel electrophoresis, flow cytometry, and fluorescence microscopy

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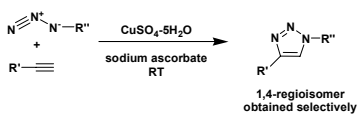
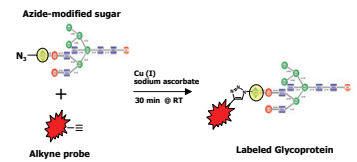
Introduction

- Glycoproteins are involved in numerous biological functions including modulation of inflammatory response, determination of biological activity, modulation of protein stability and serum half-life, and cellular communication. Modifications in glycoprotein glycan structures are associated with many disease states including cancer.
- Current analytical and cell biological methods for the characterization of glycoproteins suffer from a lack of sensitivity and selectivity and often require harsh conditions.
- Here we present versatile, highly-selective and sensitive labeling methods for the labeling and detection of specific glycoprotein subclasses, including cell surface N- and O-linked glycoproteins and intracellular O-GlcNAc modified proteins.
- Metabolic and enzymatic labeling techniques are utilized to incorporate azide residues into specific glycoprotein structures.
- The detection reaction is based upon the copper(I) catalyzed azide-alkyne [3+2] cycloaddition reaction, or "click" chemistry whereby azide-labeled glycoproteins are ligated with various fluorescent, UV-excitable, or biotinylated alkyne detection probes.

Copper(I) Catalyzed Azide-Alkyne [3+2] Cycloaddition[4]



Scheme 1 – "Click" chemistry based detection of metabolically labeled glycans[1,4]



Scheme 2 – Mutant GalT (Y289L) enzyme labeling and "Click" detection of O-GlcNAc labeled proteins[2,3,4]

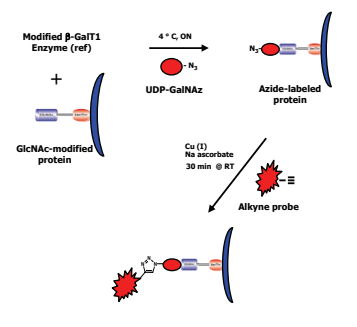
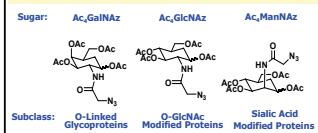


Figure 1 – Metabolic labeling and detection of glycoprotein subclasses with Click-iT™ detection reagents



- Feed cells 1-3 day HexNAz sugars
- Lyse cells and isolate proteins
- Label glycoproteins 30 min with TAMRA alkyne probe
- Precipitate proteins (remove excess label)
- Run 1-D and 2-D gels

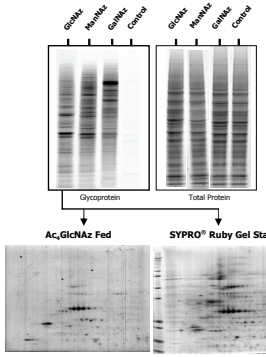


Figure 2 – Dosing Jurkat cells with unnatural azido sugars

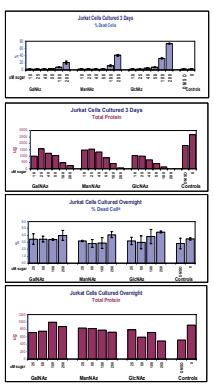


Figure 3 – Western blot comparison of Click-iT™ O-GlcNAc detection reagents with CTD110.6 anti-O-GlcNAc antibody

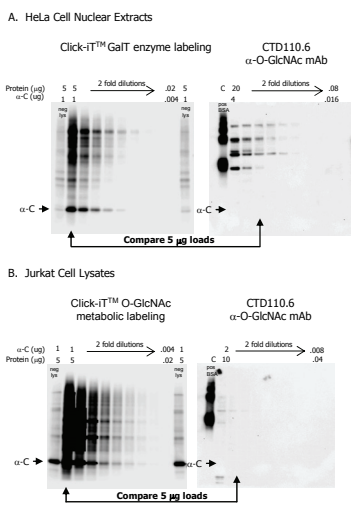


Figure 3. HeLa cell nuclear extracts (A) or Jurkat cell lysates (B) were spiked with α-crystallin and serial dilutions were run on 1-D NuPAGE® gels. (A) Left panel: proteins were labeled with Click-iT™ GalT(Y289L) and biotin-alkyne, then blotted to PVDF. Right panel: Proteins blotted to PVDF and detected with anti-O-GlcNAc antibody CTD110.6 using the Pierce O-GlcNAc detection kit. (B) Left panel: Jurkat cell lysates were metabolically labeled with $Ac_4GlcNAz$, spiked with GalT-labeled α-crystallin, and blotted to PVDF. Right panel: Jurkat cell lysates spiked with α-crystallin, blotted and detected with CTD110.6 as above. All Western blot detection was performed using protocols and reagents provided in the Pierce O-GlcNAc detection kit.

Figure 4 – Click-iT™ fluorescence gel detection of GalT (Y289L) labeled O-GlcNAc modified proteins

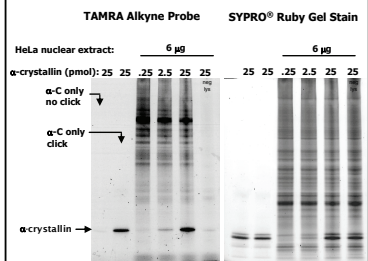


Figure 4. α-Crystallin, (2-10% O-GlcNAc-modified), and HeLa cell nuclear extract were enzymatically labeled with modified β-GalT1 enzyme. The protein was subsequently reacted with a fluorescent alkyne probe as described. The proteins were run on 1-D NuPAGE® Novex® gels. Lanes 1) 25 pmol α-crystallin without enzyme; Lane 2) 25 pmol α-crystallin; Lanes 3-5) 6 µg HeLa cell nuclear extract + 0.25, 2.5 and 25 pmol α-crystallin; Lane 6) 6 µg HeLa cell nuclear extract without enzyme + 25 pmol unlabeled α-crystallin.

Figure 5 – Multiplexing Click-iT™ O-GlcNAc detection with Pro-Q® Diamond Phosphoprotein gel stain and SYPRO® Ruby gel stain

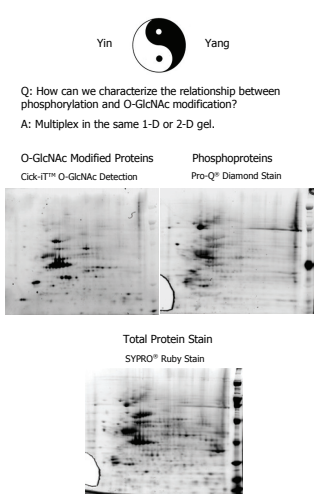


Figure 5. Jurkat cells were labeled with 30 mM $Ac_4GlcNAz$ for 3 days. Proteins were isolated and labeled with Dapoxyl-alkyne for 30 minutes. Proteins were separated on a pH 4-7 IEF strip in the first dimension and a NuPAGE® Novex® 4–12% Bis-Tris gel in the second dimension using the ZOOM® benchtop proteomics system. After UV imaging, the gel was serially stained with Pro-Q® Diamond Stain and SYPRO® Ruby Stain.

Figure 6 – High resolution "click" based staining of cells labeled with HexNAz sugars

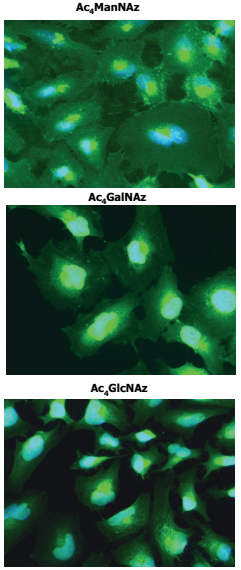


Figure 6. HeLa cells were grown in 30 mM HexNAz sugars for 24-48 hours, then fixed and permeablized. "Click" labeling reactions using fluorescent alkyne dyes (green) were performed for 15 minutes. Nuclear staining is shown in blue using DAPI.

Figure 7 – Fluorescence-based flow analysis of Jurkat cells metabolically labeled with $Ac_4ManNAz$, $Ac_4GalNAz$, or $Ac_4GlcNAz$ and "click" labeled with a 488 excitable alkyne dye: External versus external/internal staining

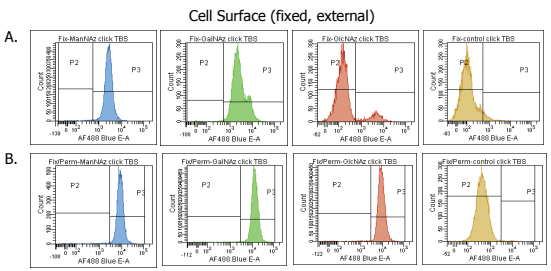


Figure 7. Jurkat cells were fed 30 µM $Ac_4HexNAz$ sugars for 3 days. In A., live Jurkat cells were labeled with fluorescent probe directly, fixed only, and analyzed by flow cytometry. In B, cells were fixed and permeabilized before labeling with probe and analyzed.

Figure 8 – Pulse /chase labeling of GalNAz fed HeLa cells demonstrating O-linked membrane glycoprotein internalization

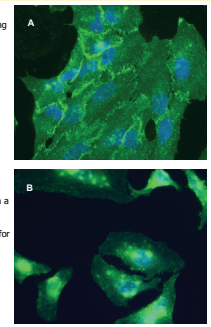


Figure 8. A) HeLa cells were cultured in MEM/10% FBS containing 30 uM GalNAz for 48 hrs, stained with a 488 excitable alkyne dye for 15 min., then fixed with 4% formaldehyde/PBS for 15 min. and stained with DAPI nuclear stain.

B) HeLa cells were cultured in MEM/10% FBS containing 30 µM GalNAz for 24 hrs then stained with a 488 excitable alkyne dye for 15 min. After staining, the media was replaced and the cells were grown for an additional 24 hrs, then fixed with 4% formaldehyde/PBS for 15 min. and stained with DAPI nuclear stain.

Figure 9 – Inside/outside three-color staining of fixed and fixed/permeablized GalNAz labeled HeLa cells

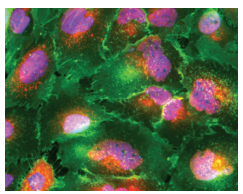


Figure 9. HeLa cells were cultured in MEM/10% FBS containing 30 µM GalNAz for 48 hrs. Cells were fixed with 4% formaldehyde/PBS for 15 minutes stained with a 488 excitable alkyne dye for 15 minutes, permeablized with 1% NP40/PBS for 15 minutes, stained with a 594 excitable alkyne dye for 15 minutes, then post-stained with DAPI nuclear stain. Cell surface staining is shown in green, golgi staining in red, and nuclear staining in pink (overlap of blue DAPI and red GalNAz).

Results and Conclusions

- Click-iT™ metabolic labeling reagents, $Ac_4GlcNAz$, $Ac_4ManNAz$, and $Ac_4GalNAz$ effectively label specific subsets of cellular glycoproteins in cultured cells as demonstrated by 1-D and 2-D gel electrophoresis, fluorescence cell staining, and flow cytometry.
- $Ac_4HexNAz$-labeled cells can be differentially labeled using two-color fluorescence staining techniques enabling the discrimination of cell surface glycoproteins from cytoplasmic/nuclear glycoproteins.
- Click-iT™ GalT(Y289L) O-GlcNAc labeling kit efficiently labels O-GlcNAc-modified proteins *in vitro* with high sensitivity and selectivity.
- Click-iT™ Western blot and gel detection of O-GlcNAc-modified proteins is highly sensitive when compared to the antibody-based detection system using CTD110.6 α-O-GlcNAc antibody.
- Pulse/chase labeling of live cells can be utilized to follow spatial and temporal changes in glycoprotein expression.

References

1. Dube D.H., Bertozzi C.R. (2003). *Curr Opin Chem Biol.* Oct;7(5):616-25.
2. Boeggeman E.E., Ramakrishnan B., Qasba P.K. (2003). *Protein Expr Purif* Aug;30(2):219-29.
3. Khidekel N., Arndt S., Lamarre-Vincent N., Lippert A., Poulin-Kirstien K.G., Ramakrishnan B., Qasba P.K., Hsieh-Wilson L.C. (2003). *J Am Chem Soc* Dec 31;125(52):16162-3.
4. Rostovtsev V.V., Green L.G., Folkin V.V., Sharpless K.B. (2002). *Angew Chem Int Ed Engl.* Jul 15;41(14):2596-9.

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