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CLICK-IT™ GLYCOPROTEIN PROFILING REAGENTS.

Click—The next generation in fluorescence labeling and detection

Fluorescence labeling and detection methods have enabled significant advancements in biomedical research by providing tools that supplant hazardous radioactive and insensitive colorimetric assays and reagents. Labeling a specific class of biomolecules in the context of a live cell or complex cell lysate, however, requires a selectivity that common reactive fluorescent probes cannot deliver. Fluorescence chemistry traditionally targets amines, sulfhydryls, and carboxylates—functional groups that are not unique to the biomolecule of interest. Indirect labeling methods that employ antibody or lectin conjugates offer the selectivity that reactive fluorescent probes lack, but this selectivity comes with several limitations. The large size of these protein conjugates severely restricts their access to intracellular compartments. Furthermore, because they are often the same size or larger than their targets, antibody and lectin conjugates potentially modify the solubility, localization, and interactions of the molecule under study. Genetically encoded fluorescent protein reporters such as Green Fluorescent Protein (GFP) and its variants circumvent the cell permeability issue by using intracellular transcription and translation machinery to label the protein, but here too the protein-sized fluorescent reporter may interfere with the normal functioning of the protein.

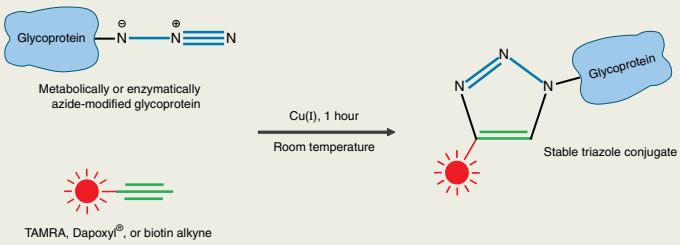

Figure 1—Click-iT™ azide/alkyne reaction.

What's needed is a chemoselective labeling technique that is compatible with complex biological systems and relatively transparent to the cell machinery. The new Click-iT™ technology employs an advanced two-step labeling method that addresses both the size of the fluorescent tag and the selectivity of the labeling reaction. Here we describe our first application of this technology: Click-iT™ glycoprotein labeling reagents and Click-iT™ Glycoprotein Detection Kits, which are based on this two-step labeling method.

In the first step, a specific class of biomolecules (in this case, glycoproteins) in an experimental sample—be it live cells, cell lysate, or protein fraction—is enzymatically labeled with an unnatural biosynthetic precursor containing a Click-iT™ azide handle. The choice of azide-containing substrate and corresponding enzyme defines the selectivity of this labeling reaction; the incorporation of the small (three-atom) azide moiety provides a bioorthogonal functional group that typically neither reacts with other cell components nor disrupts normal cell processes. If the target molecule contains a unique functional group, this first labeling step can also be accomplished nonenzymatically using a reactive azide. In the second step, molecules selectively labeled with the Click-iT™ azide handle are detected with a fluorescent or biotinylated alkyne using Cu(I)-catalyzed cycloaddition or "click" chemistry [1–4] (Figure 1). Not only is it fast, selective, and extremely efficient, but the click reaction produces a very stable covalent bond between the azide and alkyne, capable of withstanding mass spectrometry (MS) ionization.

Click-iT™ technology has far-reaching implications for a multitude of *in vivo* and *in vitro* fluorescence labeling applications. Through the selective enzymatic incorporation of a bioorthogonal Click-iT™ handle, a class of biomolecules can be tagged yet remain functional within the parameters of normal cell activity. At any point after labeling, these biomolecules can be selectively and sensitively detected using a click reaction. For example, using an azide-containing fucose analog, Sawa

and coworkers have selectively labeled fucosylated glycoconjugates via the fucose salvage pathway, detected them with an azide/alkyne click reaction using a fluorescent or biotinylated alkyne, and studied their intracellular localization by fluorescence microscopy.[5] The state-of-the-art Click-iT™ reagents allow you to completely sidestep the nonspecific binding interactions of antibodies and lectins that often plague immunolabeling experiments, as well as the potential sample destruction through β-elimination (BEMAD) and the biohazards associated with radioactivity-based detection methods.

Discover—Click-iT™ tools for glycoprotein profiling

Glycosylation is the most ubiquitous posttranslational modification of cellular proteins and yet, due to the shortcomings of existing research tools, very little is known about the role of protein glycosylation in vital cellular processes such as cell growth and differentiation, cell surface interactions, intracellular signaling, and protein stability. Harnessing the chemoselectivity of click chemistry, the new Click-iT™ glycoprotein profiling reagents provide a simple and robust two-step method for detecting and characterizing specific glycoprotein subclasses, with detection limits in the low femtomole range.

Four different Click-iT™ glycoprotein labeling reagents are available (Table 1), each providing an azide-modified sugar for biosynthetic incorporation into a specific subclass of protein glycan structures:

- Click-iT™ GalNAz metabolic glycoprotein labeling reagent, a tetraacetylated azido galactosamine for *in vivo* labeling of *O*-linked glycoproteins
- Click-iT™ ManNAz metabolic glycoprotein labeling reagent, a tetraacetylated azido mannosamine for *in vivo* labeling of cell-surface sialic acid–modified glycoproteins

Table 1—Click-iT™ glycoprotein labeling reagents.

Sample	Glycoprotein subclass	Click-iT™ labeling reagent	Cat. no.
Cultured cells	*O*-Linked	Click-iT™ GalNAz metabolic glycoprotein labeling reagent	C33365
	O-GlcNAc	Click-iT™ GlcNAz metabolic glycoprotein labeling reagent	C33367
	Sialic acid	Click-iT™ ManNAz metabolic glycoprotein labeling reagent	C33366
Pure protein, cell lysate, or protein extract	*O*-GlcNAc	Click-iT *O*-GlcNAc Enzymatic Labeling System	C33368

- Click-iT™ GlcNAz metabolic glycoprotein labeling reagent, a tetraacetylated azido glucosamine for *in vivo* labeling of intracellular *O*-linked *N*-acetylglucosamine (*O*-GlcNAc)–modified glycoproteins
- Click-iT™ *O*-GlcNAc Enzymatic Labeling System, an azido galactose for *in vitro* labeling of *O*-GlcNAc–modified glycoproteins

The Click-iT™ metabolic glycoprotein labeling reagents comprise three different tetraacetylated azido sugars, which act as biosynthetic precursors for *in vivo* labeling of three different subclasses of glycoproteins.[6–8] Cultured cells are simply incubated with the modified sugars for 2–3 days or until cells reach the appropriate density. The acetyl groups improve cell permeability of the modified sugars and are removed by nonspecific intracellular carboxylesterases. The resulting azido sugar is then metabolically incorporated into a protein glycan structure through the permissive nature of the oligosaccharide biosynthesis pathway, yielding a glycoprotein containing the Click-iT™ azide handle (Figure 2).

The Click-iT™ *O*-GlcNAc Enzymatic Labeling System supplies the key reagents for *in vitro* labeling of *O*-GlcNAc–modified glycoproteins. The →

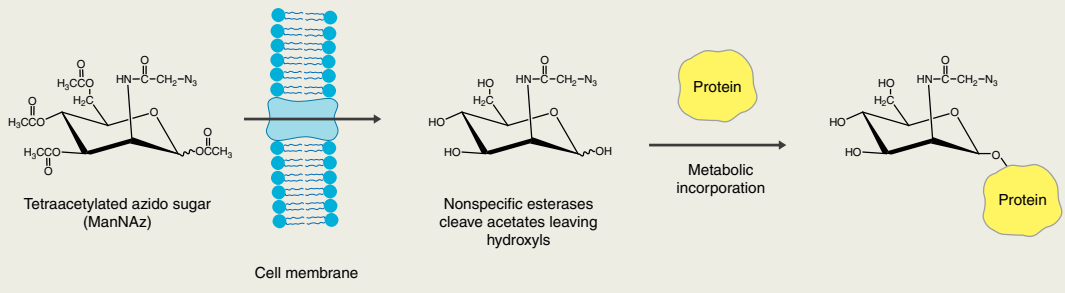

Figure 2—Metabolic incorporation of tetraacetylated azido sugars.

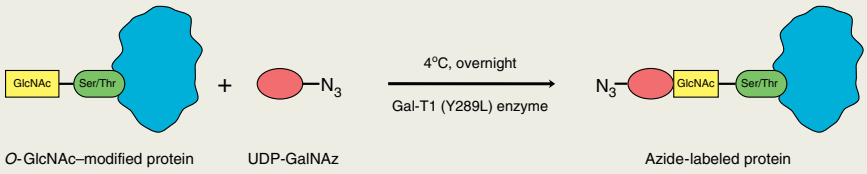

Figure 3—Enzymatic labeling of an *O*-GlcNAc–modified glycoprotein with UDP-GalNAz and the permissive mutant β-1,4-galactosyltransferase (Gal-T1 (Y289L)).

O-GlcNAc modification is a highly dynamic, intracellular regulatory modification found in all eukaryotic cells and, like phosphorylation, significantly alters target protein function.[9–12] *O*-GlcNAc–modified glycoproteins are enzymatically labeled with the Click-iT™ azide handle by utilizing the permissive mutant β-1,4-galactosyltransferase (Gal-T1 (Y289L)), which transfers azide-modified galactosamine (GalNAz) from UDP-GalNAz to *O*-GlcNAc residues on the target proteins[10] (Figure 3).

When it's time to detect the Click-iT™ azide handle, you have a choice of three different Click-iT™ Glycoprotein Detection Kits (Table 2), which provide either a fluorescent or biotinylated azide-reactive alkyne along with the critical click reaction components:

- Click-iT™ Tetramethylrhodamine (TAMRA) Glycoprotein Detection Kit
- Click-iT™ Dapoxyl® Glycoprotein Detection Kit
- Click-iT™ Biotin Glycoprotein Detection Kit

Glycoproteins labeled with a fluorescent alkyne can be directly detected in 1D or 2D gels using the appropriate excitation sources (300 nm UV illumination or 532 nm laser for the TAMRA fluorophore, 300 or 365 nm UV illumination for the Dapoxyl® fluorophore). Their spectral compatibility with total-protein, glycoprotein, and phosphoprotein gel stains is shown in Table 2. Glycoproteins labeled with the biotin alkyne can be detected on a western blot before or after probing with a primary antibody. In 1D gels and western blots, Click-iT™ technology enables the detection of a few hundred femtomoles of glycoprotein, allowing an in-depth analysis of glycosylation and its elusive cellular functions that was previously unattainable with conventional glycan labeling tools.

Publish—Truly comprehensive protein glycosylation analyses

One key advantage of the Click-iT™ method is the potential to multiplex with other detection technologies, permitting an unprecedented characterization of cellular posttranslational modifications. Used in combination with Multiplexed Proteomics® technologies, Click-iT™ labeling and detection reagents allow researchers to detect specific glycosylation subclasses together with total protein, total glycoproteins, or total phosphoproteins—all in the same gel (Table 2). With unsurpassed sensitivity over antibody-based detection methods (Figure 4), the Click-iT™ products provide a means of detecting low-abundance glycoproteins as well as glycoproteins with a small degree of glycosylation. This sensitivity may be a critical factor when analyzing the relationship between the *O*-GlcNAc modification and phosphorylation of Ser/Thr residues on key regulatory proteins.[10,13]

Table 2—Compatibility of the Click-iT™ Glycoprotein Detection Kits.

		Compatibility with detection methods			Spectral compatibility with Multiplexed Proteomics® stains		
Click-iT™ Glycoprotein Detection Kit	Cat. no.	1D or 2D gel	Western blot	Mass spectrometry	SYPRO® Ruby protein gel stain	Pro-Q® Emerald glycoprotein gel stain	Pro-Q® Diamond phosphoprotein gel stain
Click-iT™ Tetramethylrhodamine (TAMRA) Glycoprotein Detection Kit	C33370	●	●	●	●	●	
Click-iT™ Dapoxyl® Glycoprotein Detection Kit	C33371	●		●	●		●
Click-iT™ Biotin Glycoprotein Detection Kit	C33372		●	●			

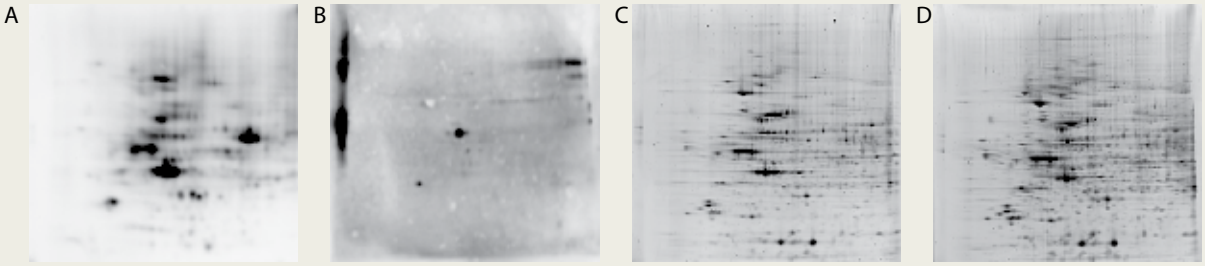

Figure 4—Comparison of Click-iT™ metabolic detection of *O*-GlcNAc–modified glycoproteins with antibody-based detection. (A) Jurkat cells were metabolically labeled with Click-iT™ GlcNAz metabolic glycoprotein labeling reagent, followed by biotinylation of the GlcNAz-labeled proteins with the Click-iT™ Biotin Glycoprotein Detection Kit. 20 mg of cell extract was separated on pH 4–7 IEF strips followed by NuPAGE® gel electrophoresis, then blotted to PVDF membrane. Biotinylated *O*-GlcNAc glycoproteins were visualized with streptavidin–horseradish peroxidase (HRP). (B) 2D western blot of 40 mg unlabeled Jurkat cell extract separated and blotted as in (A), then visualized with the anti–*O*-GlcNAc antibody CTD110.6, followed by the HRP conjugate of goat anti–mouse IgG antibody. In (A) and (B) HRP detection was performed using the protocols and reagents provided in the Pierce *O*-GlcNAc detection kit. The marker lane on the left in (B) contains 5 ng of the *O*-GlcNAc bovine serum albumin (BSA) positive control from the Pierce kit. (C) Gel of sample (A) stained with SYPRO® Ruby total protein stain. (D) Gel of sample (B) stained with SYPRO® Ruby total protein stain.

Finally, glycoproteins labeled with the Click-iT™ labeling and detection reagents are completely compatible with downstream LC-MS/MS and MALDI-MS analyses for further identification and characterization. For added convenience, we offer an *O*-GlcNAc peptide LC/MS standard (C33374) from the transcription factor CREB for LC-MS/MS and MALDI-MS analyses of the *O*-GlcNAc posttranslational modification. This peptide is also available together with its phosphorylated counterpart for use as LC/MS standards (C33373) in differential mass spectrometry–based studies of the corresponding modifications, as well as for characterizing differential β-elimination/addition conditions.

If you have questions about the Click-iT™ technology or would like to suggest ideas for the next Click-iT™ products, please send us an email at **reactions@invitrogen.com**. ■

References

1. Breinbauer, R. and Köhn, M. (2003) *ChemBioChem* 4:1147–1149.
2. Wang, Q. et al. (2003) *J Am Chem Soc* 125:3192–3193.
3. Rostovtsev, V.V. (2002) *Angew Chem Int Ed Engl* 41:2596–2599.
4. Kolb, H.C. et al. (2001) *Angew Chem Int Ed Engl* 40:2004–2021.
5. Sawa, M. et al. (2006) *Proc Natl Acad Sci U S A* 103:12371–12376.
6. Dube, D.H. et al. (2006) *Proc Natl Acad Sci U S A* 103:4819–4824.
7. Prescher, J.A. et al. (2004) *Nature* 430: 873–877.
8. Luchansky, S.J. et al. (2003) *Methods Enzymol* 362:249–272.
9. Saxon, E. and Bertozzi, C.R. (2000) *Science* 287:2007–2010.
10. Love, D.C. and Hanover, J.A. (2005) *Sci STKE* 2005:re13.
11. Ramakrishnan B. and Qasba P.K. (2002) *J Biol Chem* 277:20833–20839.
12. Khidekel, N. et al. (2003) *J Am Chem Soc* 125:16162–16163.
13. Slawson, C. (2006) *J Cell Biochem* 97:71–83.

Product	Quantity	Cat. no.
Click-iT™ GalNAz metabolic glycoprotein labeling reagent (tetraacetylated *N*-azidoacetylgalactosamine) *for *O*-linked glycoproteins* *5.2 mg*	1 each	C33365
Click-iT™ GlcNAz metabolic glycoprotein labeling reagent (tetraacetylated *N*-azidoacetylglucosamine) *for *O*-GlcNAc-modified proteins* *5.2 mg*	1 each	C33367
Click-iT™ ManNAz metabolic glycoprotein labeling reagent (tetraacetylated *N*-azidoacetyl-D-mannosamine) *for sialic acid glycoproteins* *5.2 mg*	1 each	C33366
Click-iT™ *O*-GlcNAc Enzymatic Labeling System *for *O*-linked GlcNAc glycoproteins* *10 labelings*	1 kit	C33368
Click-iT™ Biotin Glycoprotein Detection Kit *10 reactions*	1 kit	C33372
Click-iT™ Dapoxyl® Glycoprotein Detection Kit *for UV excitation* *10 reactions*	1 kit	C33371
Click-iT™ Tetramethylrhodamine (TAMRA) Glycoprotein Detection Kit *UV/532 nm excitation* *10 reactions*	1 kit	C33370
Click-iT™ *O*-GlcNAc peptide and phosphopeptide LC/MS standards *5 nmol each*	1 set	C33373
Click-iT™ *O*-GlcNAc peptide LC/MS standard (H-Thr-Ala-Pro-Thr-(*O*-GlcNAc)Ser-Thr-Ile-Ala-Pro-Gly-OH) *Theoretical Mass (M+H): 1118.50*	5 nmol	C33374