

Click-iT™ O-GlcNAc Enzymatic Labeling System

Table 1. Contents and storage information.

Material	Amount	Concentration	Storage	Stability
UDP-GalNAz, Component A	50 µg	NA	<ul style="list-style-type: none"> • 2–6°C • Desiccate 	When stored as directed, the kit is stable for at least 6 months.
Gal-T1 (Y289L), Component B	125 µL	NA	<ul style="list-style-type: none"> • 2–6°C • DO NOT FREEZE 	
Click-iT™ O-GlcNAc enzymatic labeling buffer, Component C	1 mL	2.5X in a solution containing 125 mM NaCl, 50 mM HEPES, 5% NP-40, pH 7.9	<ul style="list-style-type: none"> • ≤6°C • Avoid Freeze Thaw 	
MnCl ₂ , Component D	150 µL	100 mM	2–6°C	
α-crystallin positive control protein, Component E	250 µg	NA	2–6°C	

Number of reactions: Sufficient material is supplied for 10 reactions, based on the protocol below.

Introduction

The O-GlcNAc (O-linked N-acetylglucosamine) modification is a highly dynamic intracellular regulatory modification which like phosphorylation, dramatically alters the posttranslational functions of targeted proteins. The modification is found ubiquitously among all eukaryotes, from yeast to humans, and the enzymes responsible for the dynamic addition and removal of the modification have been well characterized. Evidence suggests that an inverse relationship may exist between O-GlcNAcylation and phosphorylation, as in some cases the modifications are found on the same Ser/Thr residues on target proteins.^{1,2}

The Click-iT™ O-GlcNAc Enzymatic Labeling System provides a highly sensitive and efficient method for the *in vitro* modification of O-GlcNAc modified proteins (Figure 1). Proteins are enzymatically labeled utilizing the permissive mutant β-1,4-galactosyltransferase (Gal-T1 (Y289L) (Figure 2) which transfers azido-modified galactose (GalNAz) from UDP-GalNAz to O-GlcNAc residues on the target proteins.^{3,4} Target proteins can then be detected utilizing one of the Click-iT™ Protein Analysis Detection Kits listed in Table 2, which are compatible with downstream mass spectrometry (MS) analyses including LC-MS/MS and MALDI MS, and Invitrogen's Multiplexed Proteomics® technologies (Table 2). Labeling and detection can be completed in less than 24 hours, and is very sensitive, detecting as little as 1 picomole of α-crystallin, a protein which is only 2–10% O-GlcNAc modified (Figure 3).

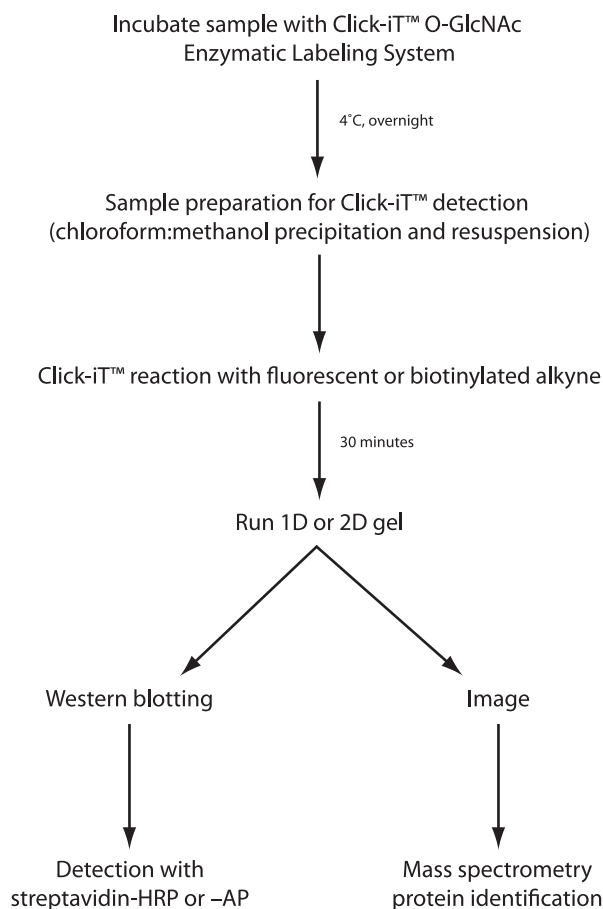


Figure 1. Workflow diagram of the Click-iT™ O-GlcNAc enzymatic labeling and detection scheme.

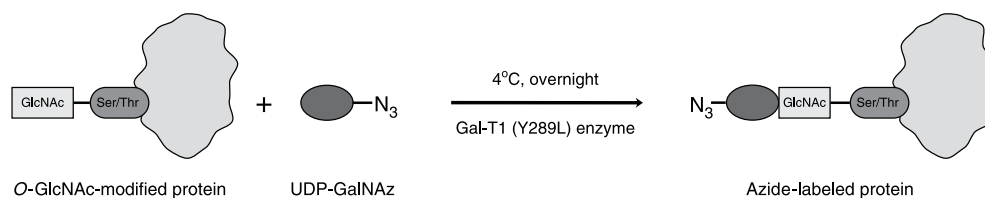


Figure 2. Enzymatic labeling of an O-GlcNAc–modified protein with UDP-GalNAz and Gal-T1 (Y289L).

Table 2. Detection and Multiplexed Proteomics® Compatibility of the Click-iT™ Protein Analysis Detection Kits.

Product	Cat. no.	Ex/Em*	Excitation Source	Detection Method	Multiplexed Proteomics® Compatibility
Click-iT™ Tetramethylrhodamine (TAMRA) Protein Analysis Detection Kit	C33370	545/580 nm	300 nm UV illumination or 532 nm laser	1D or 2D gel Western blot Mass spectrometry	<ul style="list-style-type: none"> Pro-Q® Emerald 300 glycoprotein gel stain SYPRO® Ruby protein gel stain Western detection with anti-TAMRA antibody
Click-iT™ Dapoxyl® Protein Analysis Detection Kit	C33371	370/580 nm	300 or 365 nm UV illumination	1D or 2D gel Mass spectrometry	<ul style="list-style-type: none"> Pro-Q® Diamond phosphoprotein gel stain SYPRO® Ruby protein gel stain
Click-iT™ Biotin Protein Analysis Detection Kit	C33372	Not applicable	Not applicable	Western blot Mass spectrometry	Western detection with streptavidin

*Ex/Em = Excitation and emission maxima in nm.

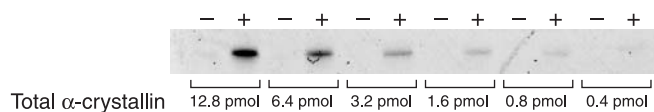


Figure 3. Dilution series of α -crystallin labeled with the Click-iT™ O-GlcNAc Enzymatic Labeling System. Detection was performed using the Click-iT™ TAMRA Alkyne Detection Reagent (+). Alternate lanes contain α -crystallin subjected to the same procedure without the addition of the Gal-T1 (Y289L) enzyme (-). Only 2% to 10% of the total α -crystallin is O-GlcNAc modified, translating to 8–40 fmol of O-GlcNAc in the 0.4 pmol band and demonstrating the sensitivity of this detection technology.

Before You Begin

Materials Required but Not Provided

- 1% SDS in 20 mM HEPES buffer, pH 7.9
- 10 mM HEPES buffer, pH 7.9
- 1% SDS in 50 mM Tris-HCl, pH 8.0
- Methanol
- Chloroform
- 18 megaOhm purified water

Preparing the UDP-GalNAz Stock Solution

- 1.1 To make a 0.5 mM solution of UDP-GalNAz, add 144 μ L of 10 mM HEPES buffer, pH 7.9 to Component A and mix well to ensure complete reconstitution.

Note: Keep the solution on ice while in use. Aliquot and freeze any unused portion at $\leq -80^{\circ}\text{C}$. When stored as directed, this stock solution is stable for up to 1 year.

Preparing the α -Crystallin Stock Solution

- 2.1 To make a 5 $\mu\text{g}/\mu\text{L}$ solution of α -crystallin (positive control), add 50 μL 1% SDS, 20 mM HEPES pH 7.9 to Component E and mix well to ensure complete reconstitution. Store the unused portion at -20°C . When stored as directed, this stock solution is stable for up to 1 year.

Experimental Protocol

Preparing the Sample

Note: In general, we suggest using soluble protein fractions or subcellular fractions that are free of cell-surface glycoproteins. Although present at low levels, cell-surface glycans may contain terminal GlcNAc residues that will also label with the Gal-T1 (Y289L) enzyme. If cell surface glycoproteins are included in the assay, PNGase F may be used to cleave cell surface glycans before labeling with the enzyme, minimizing false positives that could result from labeling cell-surface glycoproteins.

- 3.1 Aliquot 80–200 μg of lysate, tissue extract, or pure protein into a 1.5 mL microcentrifuge tube. Bring the volume of the sample up to 200 μL , if necessary, with 1% SDS, 20 mM HEPES pH 7.9.
- 3.2 Precipitate the detergents and proteins using the chloroform/methanol precipitation method below.
 - a. Add 600 μL of methanol to the 200 μL sample and vortex briefly.
 - b. Add 150 μL of chloroform and vortex briefly.
 - c. Add 400 μL of 18 megaOhm water and vortex briefly.
 - d. Centrifuge for 5 minutes at 13,000–18,000 $\times g$, then carefully remove and discard as much of the upper aqueous phase as possible while leaving the interface layer containing the protein precipitate intact.
 - e. Add 450 μL of methanol to the tube, and vortex briefly.
- 3.3 Centrifuge for 5 minutes at 13,000–18,000 $\times g$ to pellet the protein, then remove and discard the supernatant.
- 3.4 Leaving the cap open, cover the tube with a lint-free tissue and allow the pellet to air dry for 5 minutes. Samples may be air dried longer, up to overnight, but in some cases the protein may be more difficult to resolubilize following extended drying times.
- 3.5 Resuspend the protein in up to 40 μL of 1% SDS in 20 mM HEPES pH 7.9.
- 3.6 Heat at 90°C for 5–10 minutes to completely dissolve the proteins.
- 3.7 Vortex gently and cool on ice for 3 minutes.

Note: Longer cooling may precipitate the SDS. If this happens, warm the sample to room temperature and vortex briefly to resolubilize the SDS. Visually inspect the sample to see that the pellet has dissolved.

Enzymatic Labeling of Protein Sample

- 4.1 In a separate 1.5 mL tube, pipet 4 μL of α -crystallin control protein (prepared in step 2.1).
- 4.2 Set up the test reaction and the positive control reaction according to the instructions below. It is important to add the various reaction components to each tube in the correct order and perform any necessary mixing steps as specified.
- 4.3 To each tube add the following components in order: the 18 megaOhm water, followed by the labeling buffer, and the MnCl_2 as described in Table 3.

- 4.4 Vortex briefly to mix, and then briefly centrifuge to collect the contents of the tube.
- 4.5 Add UDP-GalNAz (prepared in step 1.1) to each sample. Pipet up and down to mix.
- 4.6 Remove 50 μL from the test reaction and pipet into a separate 1.5 mL microcentrifuge tube for use as an unlabeled (negative) control.
- 4.7 Add Gal-T1 (Y289L) enzyme (Component B), to each sample. Pipet up and down to mix.
- 4.8 Incubate all reactions, including the unlabeled control(s), at 4°C overnight (14 to 24 hours).
- 4.9 Store samples at –20°C until analyzed using the Click-iT™ Protein Analysis Detection Kits.

Table 3. Volumes for Click-iT™ enzymatic labeling reactions.

Reaction components	Test reaction	Positive control reaction
Protein of interest: 2–5 $\mu\text{g}/\mu\text{L}$ in 1% SDS, 20 mM HEPES pH 7.9	40 μL	—
α -crystallin control protein	—	4 μL
18 megaOhm water	49 μL	4.5 μL
Labeling buffer (Component C)	80 μL	8 μL
MnCl ₂ , 100 mM (Component D)*	11 μL	1.5 μL
UDP-GalNAz	10 μL	1 μL
	(remove 50 μL to a separate tube to serve as negative control)	
Gal-T1 (Y289L) (Component B)*	7.5 μL (to the remainder of the reaction)	1 μL
Final volume	~150 μL (after removing 50 μL for negative control)	20 μL
*Mix after the addition of the component(s).		

Sample Preparation for Click-iT™ Protein Analysis Detection

Although the following protocol prepares the sample for use in an azide/alkyne click chemistry reaction, this product does not use azide/alkyne click chemistry.

- 5.1 Chloroform/methanol precipitate the protein sample and negative control proteins, from the reaction mixtures to remove excess UDP-GalNAz (see steps 3.2 and 3.3). It is not necessary to precipitate the α -crystallin positive control reaction; it will be sufficiently diluted in the detection reaction.
- 5.2 Air dry the protein sample and negative control pellets for 5 minutes.
- 5.3 Resuspend the protein sample and negative control pellets in 50 μL of 1% SDS in 50 mM Tris-HCl, pH 8.0. Add 30 μL of 1% SDS in 50 mM Tris-HCl, pH 8.0 to the α -crystallin positive control reaction.
- 5.4 The samples are now ready to be labeled with any of the Click-iT™ detection reagents. Table 2 will help you choose the appropriate Click-iT™ Protein Analysis Detection Kit and Multiplexed Proteomics® technologies based on the analytical method to be used.

References

1. Sci STKE 312, re13 (2005); 2. J Cell Biochem 97, 71 (2006); 3. J Biol Chem 277, 20833 (2002); 4. J Am Chem Soc 125, 16162 (2003); 5. J Am Chem Soc 125, 3192 (2003).

Product List Current prices may be obtained from our website or from our Customer Service Department.

Cat. no.	Product Name	Unit Size
C33368	Click-iT™ O-GlcNAc Enzymatic Labeling System *for O-linked GlcNAc glycoproteins* *10 labelings*	1 kit
<i>Related Products</i>		
A6397	anti-tetramethylrhodamine, rabbit IgG fraction *1 mg/mL*	0.5 mL
C10102	Click-iT™ AHA (L-azidohomoalanine) *for nascent protein synthesis*	5 mg
C21852	CandyCane™ glycoprotein molecular weight standards *200 gel lanes*	400 µL
C33365	Click-iT™ GalNAz metabolic glycoprotein labeling reagent (tetraacetylated N-azidoacetylgalactosamine) *for O-linked glycoproteins* *5.2 mg*	1 each
C33366	Click-iT™ ManNAz metabolic glycoprotein labeling reagent (tetraacetylated N-azidoacetyl-D-mannosamine) *for sialic acid glycoproteins* *5.2 mg*	1 each
C33367	Click-iT™ GlcNAz metabolic glycoprotein labeling reagent (tetraacetylated N-azidoacetylglucosamine) *for O-GlcNAc-modified proteins* *5.2 mg*	1 each
C33370	Click-iT™ Tetramethylrhodamine (TAMRA) Protein Analysis Detection Kit *UV/532 nm excitation* *10 reactions*	1 kit
C33371	Click-iT™ Dapoxyl® Protein Analysis Detection Kit *for UV excitation* *10 reactions*	1 kit
C33372	Click-iT™ Biotin Protein Analysis Detection Kit *10 reactions*	1 kit
C33373	Click-iT™ O-GlcNAc peptide and phosphopeptide LC/MS standards *5 nmol each*	1 set
C33374	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
M33305	Multiplexed Proteomics® Phosphoprotein Gel Stain Kit #1 *with 1 L each of Pro-Q® Diamond (P33300) and SYPRO® Ruby (S12000) gel stains*	1 set
M33306	Multiplexed Proteomics® Phosphoprotein Gel Stain Kit #2 *with 200 mL each of Pro-Q® Diamond (P33301) and SYPRO® Ruby (S12001) gel stains*	1 set
M33307	Multiplexed Proteomics® Glycoprotein Gel Stain Kit *with 1 L each of Pro-Q® Emerald 300 and SYPRO® Ruby (S12000) gel stains*	1 kit
P33350	PeppermintStick™ phosphoprotein molecular weight standards *200 gel lanes*	400 µL
P21855	Pro-Q® Emerald 300 Glycoprotein Gel Stain Kit *with SYPRO® Ruby protein gel stain* *10 minigels*	1 kit
P33300	Pro-Q® Diamond phosphoprotein gel stain	1 L
P33301	Pro-Q® Diamond phosphoprotein gel stain	200 mL
P33302	Pro-Q® Diamond phosphoprotein gel stain *bulk packaging*	5 L
R33200	EZQ® Protein Quantitation Kit *2000 assays*	1 kit
S12000	SYPRO® Ruby protein gel stain	1 L
S12001	SYPRO® Ruby protein gel stain	200 mL
S21900	SYPRO® Ruby protein gel stain *bulk packaging*	5 L

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