

Glycoprotein Capture using IsoTaG Cleavable Probes

Glycosylation is a protein post-translational modification (PTM) that generates extreme proteomic diversity and contributes to a plethora of cellular functions. Protein glycosylation can have an enormous variety of biological consequences, reflecting the molecular diversity encoded in glycan structures. This same structural diversity has imposed major challenges on the development of methods to study the intact glycoproteome.

While there has been much interest in profiling the intact glycoproteome, the complexity of glycoproteoforms (and, more broadly, all proteoforms) remains challenging to completely define. Mass spectrometry (MS) is commonly employed for characterization of complex proteomic samples. A popular strategy for protein identification is the bottom-up shotgun proteomics approach. In this method, a mixture of proteins is subjected to proteolytic digestion, the resulting peptides are separated by LC and detected by MS, and their parent proteins are inferred from the assigned peptide sequences. To convert MS data acquired from proteolytic digests into protein identifications, tandem MS can be used to obtain sequence information for individual peptides, followed by comparison to an *in silico* proteolytic digest of an organism's proteome. Typically, only the most abundant peptides are selected for fragmentation, whereas data for those peptides in relatively low quantities are not obtained. An inherent problem in shotgun proteomics is identifying proteins of low abundance, such as biomarkers for disease states, against a background of proteins whose concentrations can span up to 12 orders of magnitude.

To address the unique challenges of global characterization of the intact glycoproteome, a mass-independent chemical glycoproteomics platform, termed "isotope targeted glycoproteomics" or IsoTag was developed by the Carolyn Bertozzi group. The platform comprises four central components: (i) metabolic labeling with a chemically functionalized glycan, (ii) chemical tagging and enrichment using an isotopic recoding affinity probe, (iii) directed tandem MS, and (iv) targeted glycopeptide assignment.

In traditional proteomics approaches, tandem MS is performed on the most abundant species in the full-scan mass spectra to the exclusion of lower-abundance species (Fig 1). In contrast, IsoTaG enables mass-independent, targeted glycoproteomics (Fig 1). Glycopeptides displaying the isotopic signature are computationally detected by the pattern-searching algorithm¹, which produces an inclusion list of m/z values and retention times (± 2 -min window) for ions bearing

isotopically recoded envelopes. Femtomole quantities of tagged proteins at a signal-to-noise ratio of 2.5:1 are detectable with the pattern-recognition algorithm¹. Owing to the versatility of the algorithm¹, species up to 12 kDa that have incorporated multiple tags are also directed to the inclusion list and have an equal chance of selection for tandem MS.

¹Palaniappan, K.K., et al. (2011) Isotopic signature transfer and mass pattern prediction (IsoStamp): an enabling technique for chemically-directed proteomics. ACS Chem. Biol. 6, 829–36.

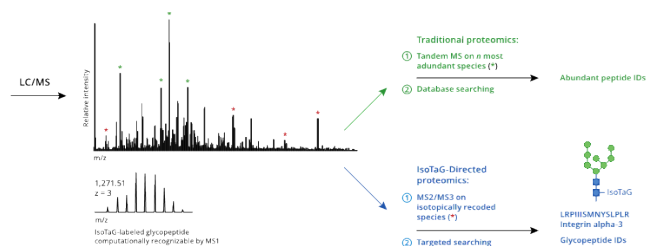


Figure 1. Traditional proteomics and Iso-Tag-directed proteomics workflow

IsoTaG is performed first by the isotopic recoding and enrichment of metabolically labeled glycoproteins followed by directed tandem MS (MS2 or MSn) analysis and intact glycopeptide assignment. Isotopic recoding is accomplished by metabolic labeling of cell or tissue samples with azide- or alkyne-functionalized sugars, followed by chemical conjugation with a biotin probe bearing a unique isotopic signature. Some examples of sugar labels include peracetylated N azidoacetylmannosamine (Ac₄ManNAz), which is converted to the corresponding azidosialic acid (SiaNAz), and peracetylated N-azidoacetylgalactosamine (Ac₄GalNAz), which is metabolized to label glycans possessing N-acetylglucosamine (GlcNAc) or N-acetylgalactosamine (GalNAc).

In order to perform isotopic tagging, two cleavable IsoTaG probes encoded by zero [M] and two [M + 2] deuterium atoms are used. Probes with different encoding can be provided by Click Chemistry Tools though custom synthesis. The IsoTaG probes with zero and two deuterium atoms [M, M + 2] can be used in different proportions: 1:1, 1:2, 1:3 and 1:4. Pattern recognition with an isotopic ratio of 1:3 showed the highest fidelity.

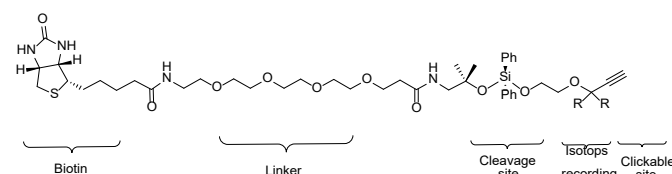


Figure 2. Cleavable IsoTaG probe encoded by zero deuterium atoms [M] (R = H) and two deuterium atoms [M+2] (R = D)

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The isotopic signature serves as a computationally recognizable full-scan MS reporter. A computational algorithm, termed isotopic signature transfer and mass pattern prediction - IsoStamp, for the detection of recoded species in full-scan mass spectra was also developed by the Carolyn Bertozzi group¹. IsoStamp compares observed and predicted isotopic envelopes to identify chemically tagged species in full-scan mass spectra.

IsoTag has the potential to enhance any proteomics platform that employs chemical labeling for targeted protein identification, including isotope-coded affinity tagging, isobaric tagging for relative and absolute quantitation, and chemical tagging strategies for posttranslational modification.

Materials Required

- Streptavidin Agarose Resin
- DADPS H2 Biotin Alkyne [M] and DADPS D2 Biotin Alkyne [M+2] (for azide sugars) (<https://clickchemistrytools.com/product/-dadps-h2-d2-biotin-alkyne-pack/>)
- DADPS H2 Biotin Azide [M] and DADPS D2 Biotin Azide [M+2] (for alkyne sugars) (<https://clickchemistrytools.com/product/-dadps-h2-d2-bitoin-azide/>)
- BTAA (<https://clickchemistrytools.com/product/btaa/>)
- Copper (II) Sulfate pentahydrate (<https://www.sigmaaldrich.com/catalog/product/sigald/c7631?lang=en®ion=US>)
- Sodium ascorbate (<https://www.sigmaaldrich.com/catalog/product/sigma/a4034?lang=en®ion=US>)
- Azide or alkyne sugar for labeling (<https://clickchemistrytools.com/product-category/metabolic-labeling-reagents/glycosylation/>)
- 5% Formic acid in water
- Protease inhibitor
- Tris
- SDS
- DTT
- Urea
- Iodoacetamide
- Acetonitrile
- Trypsin (mass spectrometry grade)
- TFA
- Methanol
- Chloroform
- ZipTip® C18 (https://www.emdmillipore.com/US/en/product/Zip-Tip-with-0.2-L-C18-resin,MM_NF-ZTC18M008 and https://www.emdmillipore.com/US/en/product/Zip-Tip-with-0.6-L-C18-resin,MM_NF-ZTC18S008)

Important: Any solution directly involved in click reaction should not contain copper chelators (EDTA, EGTA, etc.) and reducing agents (DTT, 2-mercaptoethanol, TCEP, etc.).

Additional information

- Concentration of the biotinylation reagent in a click labeling reaction may range from 2 μ M to 60 μ M. Concentrations below or above this range are also possible, and should be optimized for the specific application. We recommend starting with a 40 μ M concentration of the biotinylation reagent, and titrating this amount down in the case of high background or up in the case of low reaction efficiency.
- The isotopic ratio (H2:D2 probes or [M]:[M+2] ratio) is application dependent. Pattern recognition with an isotopic ratio of 1:3 showed the highest fidelity. However, investigators may wish to determine the optimal ratio for each application.

Material Preparation

- **DADPS Biotinylation reagent:** Dissolve 1 mg of each DADPS Biotinylation reagents in 1 mL of DMSO. Mix these H2 and D2 DADPS Biotin probes at desired ratio. Isotopic ratio of 1:3 (100 μ L of H2 and 300 μ L of D2) can be used for initial experiments. After use, store unused detection reagent stocks at -20°C for up to 1 year.

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- **Copper Catalyst (100 mM CuSO₄, 200 mM BTAA):** Weight out 250 mg of Copper (II) Sulfate Pentahydrate and 860 mg of BTAA, add 10 mL of water, vortex to dissolve completely.
- **Sodium Ascorbate:** Dissolve 20 mg of sodium ascorbate in 100 µL of deionized water. Vortex until completely dissolved. Sodium ascorbate solution is susceptible to oxidation. We recommend always using freshly prepared solution of sodium ascorbate.

Metabolic Labeling

This protocol details the preparation of metabolically labeled cell lysates for IsoTaG enrichment. The following is a standard protocol for preparation of azido- or alkynyl-sugar labeled cell lysates and conditioned media. However, investigators may wish to determine the optimal concentration of metabolic reagent as well as labeling time individually for each cell type on a small-scale first. Metabolic labeling is critical for successful glycoproteomics and should be carefully assessed for each cell line of interest prior to MS analysis. Metabolic labeling of glycoproteins is dependent on three considerations: first, the sugar precursor selected for labeling; second, access of the sugar precursor to the cell line; and third, glycosylation pathways activated in the cell line. Options for sugar precursor selection include Ac₄ManNAz, Ac₄GalNAz, Ac₄GlcNAz, and Ac₄ManNAI, among others. Ac₄ManNAz and Ac₄ManNAI are reporters of sialic acid. Ac₄GalNAz and Ac₄GlcNAz are able to access an intracellular epimerase pathway and are incorporated into O-GalNAc, O-GlcNAc, and N-GlcNAc sites. The efficiency of metabolic labeling is dependent on access of the sugar precursor to the cell line, and testing with several sugar precursors may be necessary for best results. Finally, sugars with access to multiple metabolic endpoints (e.g., Ac₄GalNAz) will be incorporated to a set of glycoproteins that are reflective of the glycosylation pathways currently activated in the cell line. In general, human cell lines are permissive to the sugar precursor, and cell counts of 10⁷-10⁸ (equivalent to 1-3 mg of total protein) are sufficient inputs for this protocol. In the event that metabolic labeling is prohibitive, alternate options to install biorthogonal handles to the sample of interest may be considered (e.g., enzymatic labeling).

- 1.1 Prepare a 500 mM stock solution of metabolic labeling reagent in DMSO to make a 1,000X to 5,000X stock solution. Aliquot and store any unused reagent at -20°C. When stored as directed, this stock solution is stable for up to 1 year.

- 1.2 Seed the appropriate number of cells so they are fully confluent after a 4-day incubation period. To the media, add metabolic labeling reagent (50-100 µM) or DMSO vehicle control containing Ac₄GalNAc. Incubate the cells at 37°C for 48 hours in a humidified, 5% CO₂ incubator. This procedure yields 3-5 mg of protein.

Note: for analysis of cellular and membrane incorporation only, the current incubation period is sufficient and the user may skip to Preparation of Cell Lysates.

- 1.3 Aspirate the media and wash the cells with PBS. Then, change the media to the one containing 100 µM glycan metabolite without FBS additive and incubate cells at 37°C, 5% CO₂ for an additional 48 hours.

Preparation of Conditioned Media (Optional)

- 2.1 Transfer the conditioned media to centrifuge tubes.
- 2.2 Clear the conditioned media by centrifugation (150 g, 3 minutes).
- 2.3 Repetitively spin concentrate the clarified media to a final volume of 500 µL (Amicon® spin filter, 3,700 g, 15-30 minutes per spin, approximately five spins required). Discard the flow through appropriately.
- 2.4 Wash the concentrated proteins with 0.1% triton X-100 in PBS (3 × 15 mL) by centrifugal filtration (3,700 g, 15-30 minutes) to remove excess labeling reagent.
- 2.5 Transfer the residue to a 2 mL microcentrifuge tube. The concentrated conditioned media may be stored for at least 12 months at -80°C.

Harvesting Suspension Cells

- 3.1 Pellet the cells by centrifugation at 400 g for 5 minutes. Discard the supernatant.
- 3.2 Resuspend the cell pellet in PBS by gently pipetting up and down using 5 mL of PBS for cells from a 100 mm dish or 1 mL of PBS per well for cells from a 6-well plate.
- 3.3 Pellet the cells by centrifugation at 400 g for 5 minutes. Discard the supernatant.

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- 3.4 Repeat the PBS wash 2 more times (steps 3.2–3.3) for a total of 3 washes to remove serum.
- 3.5 Pellet the cells by centrifugation at 400 g for 5 minutes. Discard the supernatant. The cell pellet can be used directly in step 5.1 or flash frozen and stored at -80°C until use.
- 3.6 For Suspension Cells, pellet them by centrifugation at 400 g for 5 minutes, wash 3 times with PBS, discard supernatant, and use directly in step 5.1 or flash freeze and store the pellet at -80°C .

Harvesting Adherent Cells

Note: If analyzing cell surface proteins, do not use trypsin to detach cells, because trypsin cleaves cell surface proteins. The cells can be lysed directly in the culture dish, or, if desired, use a non-enzymatic dissociation buffer or a cell scraper, and pellet the cells. If not used immediately in step 5.1 the cell lysate can be frozen and store it at -80°C until use.

- 4.1 Cells can be harvested with cell detaching solution (EDTA-free trypsin or non-enzymatic) or proceed immediately to the cell lyse step for preparing samples for the click reaction.
- 4.2 After removing the medium or cell detaching solution, wash the cells three times with PBS and discard supernatant.

Preparation of the Cell Lysates

Do not use DTT, TCEP, or β -mercaptoethanol because they will reduce the azide. Do not use EDTA or any other chelators because they will inhibit the click reaction.

The following is a standard protocol for preparation of cell lysates. However, investigators may wish to determine the optimal protocol (e.g., lysis protocol with cellular fractionation) individually for target proteins localization². Cell lysate preparation is critical for successful glycoproteomics and should be carefully assessed for each target protein subset prior to MS analysis.

- 5.1 Prepare the lysis buffer by adding protease and phosphatase inhibitors at appropriate concentrations to 1% SDS in 50 mM Tris-HCl, pH 8.0. Alternate lysis protocols (e.g., RIPA buffer, high-salt extraction) are compatible with downstream enrichment.

Note: Protease and phosphatase inhibitors are optional but recommended to ensure sample integrity.

- 5.2 For adherent cells, add 500 μL lysis buffer per 100 mm plate or 200 μL lysis buffer per well of a 6-well plate to the labeled cells. If adding the lysis buffer directly to the plate, tap or rotate the plates so the lysis buffer covers the bottom surface of the plate.

For suspension cell pellet, add 50 μL lysis buffer per 1×10^6 cells.

- 5.3 Incubate the cells for 15–30 minutes on ice, and then tilt the plates and pipet the lysate into a 1.5 mL microcentrifuge tube. If the lysis buffer does not contain Benzonase[®] endonuclease, the lysate may be very viscous due to the DNA from the lysed cells.

If using Benzonase[®] endonuclease, proceed to step 5.5.

- 5.4 Sonicate the lysate with a probe sonicator to solubilize the proteins and disperse the DNA.
- 5.5 Gently vortex the lysate for 5 minutes.
- 5.6 Centrifuge for 10 minutes at 13,000–20,000 g at 4°C .
- 5.7 Transfer the supernatant to a clean tube and label as the “soluble fraction”.
- 5.8 Measure the protein concentration of the three fractions (conditioned media, soluble, and insoluble) by BCA or any other assay, and normalize protein concentration to 5 mg/mL.

If the protein concentration is lower than 5 mg/mL, normalize to the lower value and adjust the volumes used in following steps accordingly.

- 5.9 The protein sample is now ready for the click labeling reaction with IsoTag Biotin Probes.

Click Labeling Reaction with IsoTag Biotin Probe

This protocol provides guidelines for the click reaction using 600 μL of cell lysate. However, it can be suited for smaller or larger volumes with adjustments of volumes up or down accordingly. The concentration of the biotinylation reagent in this protocol is set to 40 μM . The concentrations of biotinylation reagent may range from 2 μM to 60 μM ; concentrations below or above this range are also possible, and should be optimized for each sample type. If a higher concentration of biotin probe required, increase only the volume of IsoTag reagent in the click labeling reaction leaving all other amounts unchanged.

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6.1 For each alkyne-modified protein lysate sample, add the following to a 2.0 mL microfuge tube, then vortex briefly to mix:

- 20 μ L IsoTag DADPS Biotinylation Reagent (mixed at desired ratio).
- 50 μ L Copper Catalyst.
- 30 μ L Sodium Ascorbate.

6.2 Vortex the tube briefly to mix. The pale blue solution turns colorless after the addition of the Sodium Ascorbate.

6.3 Add the solution from Step 6.2 to 600 μ L of cell lysate.

6.4 Vortex continuously or rotate end-over-end for one hour at room temperature.

6.5 Add the labeling reaction to 2.8 mL of cold (-20°C) methanol, 0.7 mL of Chloroform and 2.1 mL of water. Cool it to -20°C for 1 hour.

Note: Cold (-20°C) acetone can be used instead of methanol: chloroform: water mixture.

6.6 Centrifuge for 10 minutes at 13,000-20,000 g, carefully remove upper aqueous layer without disturbing interface layer containing proteins.

6.7 Add 700 μ L of methanol, vortex briefly.

6.8 Centrifuge for 5 minutes at 13,000-20,000 g to pellet protein. Carefully remove and discard supernatant.

6.9 Open the lid of the tube and allow protein pellet to air dry. Do not over dry the pellet!

Binding Biotinylated Proteins to Streptavidin Agarose Resin

7.1 Resuspend air-dried protein pellets (from Step 6.9) in 800 μ L of resuspension buffer (50 mM Tris, 150 mM NaCl and 1% SDS). Other denaturing pulldown buffers (for example 6 M urea, 2 M thiourea, and 10 mM HEPES) are also compatible with downstream analysis.

7.2 Solubilize the proteins by brief sonication with a probe tip sonicator. A clear solution will result, indicating a fully solubilized pellet. Save an aliquot (50 μ L, "input") for further analysis.

7.3 Wash the streptavidin agarose resin (200 μ L of slurry per sample) with PBS (2 \times 1 mL) and with resuspension buffer (2 \times 1 mL) by either

vacuum filtration or centrifugal force (1,000 g, 3 min).

7.4 Resuspend the slurry with resuspension buffer (200 μ L) and add the slurry to the solubilized proteins.

If Western blot analysis indicates incomplete capture of biotinylated proteins, increase slurry amount in future.

7.5 Incubate the resulting mixture for 2 hours at room temperature with end-over-end rotation.

7.6 Pellet the resin by centrifugation (1,000 g, 3 min).

7.7 Separate the supernatant. Save an aliquot (100 μ L, "flow-through") for further analysis.

7.8 Wash the resin with resuspension buffer (1 \times 1 mL), 1% SDS in PBS (2 \times 1 mL), and PBS (2 \times 1 mL) in succession. Pellet the resin by centrifugation (1,000 g, 3 min) between washes, and discard the wash.

Note: Stringency of washing may be increased (e.g., 8 M urea washes) if high protein background is observed by MS or Western blot.

7.9 Resuspend the washed resin in PBS (200 μ L). Save an aliquot (10 μ L, "capture") of the slurry for further analysis.

On Bead Trypsin Digestion of Enriched Glycoproteins

8.1 Add to the resin 10 μ L of 1 M DTT, vortex briefly.

8.2 Heat to 70°C on a heat block for 15 minutes, and then cool to room temperature for 15-30 minutes.

8.3 Centrifuge resin for 5 minutes at 1,000 g, aspirate and discharge the supernatant taking care not to aspirate the resin.

8.4 Add 1 mL of 40 mM iodoacetamide solution to the resin, vortex to resuspend the resin, incubate the reaction in the dark for 30 minutes at room temperature.

8.5 Pellet the beads by centrifugation (1,000 g, 3 min) and wash once with PBS (1 mL).

8.6 Resuspend the resin in 300 μ L of digestion buffer (100 mM Tris pH 8.0, 2 mM CaCl₂, 0.5 M Urea).

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- 8.7 Add 3 µg of Trypsin from a stock solution. Gently mix the slurry, incubate at 37°C for 6-12 hours with over-the-end rotation.

Note: Alternative proteases and trypsin digestion procedures are also compatible with this protocol and may be used to increase glycopeptide coverage.
- 8.8 Pellet the resin by centrifugation (1,000 g, 3 minutes), and collect the supernatant digest.
- 8.9 Wash the resin with PBS (1 x 200 µL) and water (2 x 200 µL).
- 8.10 Combine the washes with the supernatant digest to form the "Trypsin fraction".
- 8.11 Concentrate the "Trypsin fraction" to dryness using a SpeedVac set to 40°C.
- 8.12 The "Trypsin fraction" may be stored for at least 12 months at -80°C.

Cleavage and Recovery of Glycopeptides

- 9.1 Cleave the probe by adding 5% formic acid-water (200 µL) to the resin and incubate for 30 minutes at room temperature with end-over-end rotation.
- 9.2 Pellet the resin by centrifugation (1,000 g, 3 min) and collect the supernatant.
- 9.3 Repeat Step 9.1 a second time.
- 9.4 Wash the resin with 50% acetonitrile-water + 1% formic acid (2 x 400 µL).
- 9.5 Combine the washes with the cleaved supernatant to form the "Cleavage fraction".
- 9.6 Concentrate the "Cleavage fraction" to dryness using a SpeedVac heated to 40°C. Store at -20°C until desalting and MS analysis.
- 9.7 For long term storage, the "Cleavage fraction" may be stored for at least 12 months at -80°C.

Preparation of Digest for Mass Spectrometry Analysis

The following protocol is provided for desalting using ZipTip® C18. Sample recovery for typical peptides is usually >85%, but could be as low as 35% for hydrophilic peptides. Other protocols for sample preparations for MS also can be used.

- 9.1 Resuspend the dried "Trypsin fraction" and "Cleavage fraction" in 1% formic acid in water (25 µL). Gently vortex the sample and briefly centrifuge to collect all liquid at the bottom.
- 9.2 Wet the tip by aspirating 10 µL of 50% ACN in water and then discarding solvent. Repeat once.
- 9.3 Equilibrate tip by aspirating 10 µL of 0.1% aqueous formic acid and discarding solvent. Repeat twice.
- 9.4 Load the peptides by pipetting the sample 10-15 times. Carefully pipette the sample such that air bubbles do not pass through the C18 tip. Air bubbles decrease loading capacity.
- 9.5 Wash the pipette tip with 1% formic acid in water (50 µL) and discard the eluent.
- 9.6 Slowly aspirate 25µL of 50% ACN:Water with 1% formic acid. Repeat twice.
- 9.7 Concentrate the sample using a SpeedVac heated to 40°C. Store at -20°C until analysis by LC/MS. For long-term storage, the desalted samples may be stored for at least 12 months at -80°C.

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References

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4. Weerapana, E., et al. (2010). Quantitative reactivity profiling predicts functional cysteines in proteomes. *Nature*, **648**: 790–5.
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Troubleshooting

Problem	Possible Cause	Solution
Low yield of enriched proteins	Inefficient protein capture or low abundance of azide-tagged proteins	Increase lysate concentration (use more cells) or pre-enrich the proteins (e.g., soluble lysate, membrane lysate, lectin enrichment, etc.) Confirm peptide recovery by measuring A280 after digestion
	Inefficient digestion of resin-bound proteins	Use high quality trypsin
High background with unlabeled control cells	Insufficient washing of resin	Increase column washes Use only high purity reagents Prepare filtered buffers fresh Ensure proper preparation of copper catalyst solution
Signal suppression during MS analysis	SDS contamination in digest	Wash the resin thoroughly after the SDS wash with another buffer such as 8M urea and 20% acetonitrile to remove all traces of SDS detergent