

Video Article

A Quantitative Glycomics and Proteomics Combined Purification Strategy

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Abstract

There is a growing desire in the biological and clinical sciences to integrate and correlate multiple classes of biomolecules to unravel biology, define pathways, improve treatment, understand disease, and aid biomarker discovery. *N*-linked glycosylation is one of the most important and robust post-translational modifications on proteins and regulates critical cell functions such as signaling, adhesion, and enzymatic function. Analytical techniques to purify and analyze *N*-glycans have remained relatively static over the last decade. While accurate and effective, they commonly require significant expertise and resources. Though some high-throughput purification schemes have been developed, they have yet to find widespread adoption and often rely on the enrichment of glycopeptides. One promising method, developed by Thomas-Oates *et al.*, filter aided *N*-glycan separation (FANGS), was qualitatively demonstrated on tissues. Herein, we adapted FANGS to plasma and coupled it to the individuality normalization when labeling with glycan hydrazide tags strategy in order to achieve accurate relative quantification by liquid chromatography mass spectrometry and enhanced electrospray ionization. Furthermore, we designed new functionality to the protocol by achieving tandem, shotgun proteomics and glycosylation site analysis on hen plasma. We showed that *N*-glycans purified on filter and derivatized by hydrophobic hydrazide tags were comparable in terms of abundance and class to those by solid phase extraction (SPE); the latter is considered a gold standard in the field. Importantly, the variability in the two protocols was not statistically different. Proteomic data that was collected in-line with glycomic data had the same depth compared to a standard trypsin digest. Peptide deamidation is minimized in the protocol, limiting non-specific deamidation detected at glycosylation motifs. This allowed for direct glycosylation site analysis, though the protocol can accommodate ¹⁸O site labeling as well. Overall, we demonstrated a new in-line high-throughput, unbiased, filter based protocol for quantitative glycomics and proteomics analysis.

Video Link

The video component of this article can be found at <http://www.jove.com/video/53735/>

Introduction

In the field of proteomics, filter aided sample preparation (FASP) has been widely adopted for its ability to minimize the amount of starting material, decrease sample preparation artifacts, and maximize sample throughput¹. However, such a method has yet to emerge and gain traction for the field of glycomics. Development of high-throughput, quantitative workflows are needed because of the integral role of glycosylation in biological defense and its modulation by cancer or diseases^{2,3}. In mammals, *N*-glycans are composed of repeating saccharide units (hexoses (Hex), hexosamines (HexNac), sialic acids (NeuAc), and fucoses (Fuc)), which decorate a core structure (Hex₃HexNac₂)⁴ covalently bound to asparagine. Though the glycospace is considerably large when isomers are counted (>10¹²), it is quite small on a composition basis and molecular weights typically range from 1,000-8,000 Da⁵. The compositional homogeneity of the class and the hydrophilicity of glycans pose unique challenges to purification, separation, and mass spectrometry (MS) workflows⁶.

Traditionally, *N*-glycans are digested from proteins or peptides by peptide-*N*-glycosidase F (PNGase F) and then enriched by lectin affinity chromatography⁷, captured by hydrazide beads⁸, or purified via solid phase extraction (SPE)^{9,10}. While these methods are all highly effective, they introduce extra steps for desalting and limit the number of samples simultaneously processed. Over the last decade, a number of high-throughput platforms for glycomics have been proposed. Kim *et al.* published a semi-automated method using a vacuum-operated, SPE 96-well plate¹¹. Alternatively, an affinity-filter method (*N*-glyco-FASP) was developed by the Mann group, which required the initial derivatization of the filter with a composite of lectins¹². Lastly, the Thomas-Oates group proposed a semi-quantitative method, the Filter Aided *N*-Glycan Separation (FANGS), which exploited the narrow composition size of the glycospace¹³. Relying on molecular weight cut-off filters, small contaminants were first washed to waste and then the *N*-glycans were digested and eluted. Deglycosylated proteins remain on the filter in this protocol and can be subjected to in-line FASP.

Identification and quantification of glycans by electrospray ionization (ESI) MS requires off-line separations for (partial) resolution of isomers and derivatization for detection of low-abundant species. Labeling in the individuality when normalizing with glycan hydrazide tags strategy confers compatibility with reverse-phase liquid chromatography (RPLC)^{14,15}. The 4-phenethyl-benzohydrazide (P2PGN) hydrophobic tag mediates the hydrophilicity of the glycans, enhancing ionization by, on average, four-fold¹⁶. Though other techniques, such as permethylation¹⁷ or amine-

reactive tagging chemistries¹⁸, offer similar advantages, in the hydrazide reaction, glycans are reacted 1:1 stoichiometrically in facile conditions. Relative quantification is achieved by tandem analysis of samples derivatized with native (NAT) or ¹³C₆ stable isotope labels (SIL).

The following method evolves FANGS for plasma applications and couples it to the P2GPN hydrophobic tag for accurate relative quantification. Furthermore, it was designed to perform shot-gun proteomics, deamidation profiling, and quantitative glycomics on a single aliquot of sample, without compromising the integrity of the analyses.

Protocol

1. Protein and Glycoprotein Denaturation and Alkylation

- For standard preparations, load 2.5 μ l of a 0.1 μ g/ μ l solution of glycoprotein (e.g. Fetuin or RNase B) onto a 30 kDa or 10 kDa molecular weight (MW) cut-off filter.
 - For biological plasma samples, check the protein concentration by a standard Bradford¹⁹ or bicinchoninic acid²⁰ (BCA) protein assay. Dilute the plasma, if necessary, with 100 mM ammonium bicarbonate (NH₄HCO₃) in H₂O (PNGase Digest Buffer) at a pH of ~7 to contain between 10-100 mg/ml total protein. Load 2.5 μ l plasma (25-250 μ g protein) onto a filter.
Note: This protocol has only been tested on plasma from blood samples collected in the presence of ethylenediaminetetraacetic acid (EDTA).
- Add 2 μ l of 1 M Dithiothreitol Solution (DTT) to each sample in the filter.
- Dilute the sample with 200 μ l PNGase digest buffer.
- Cap the filter sample tube and lightly vortex, being careful not to disturb the filter from its seat. Incubate the sample at 56 °C for 30 min to denature the proteins and glycoproteins.
- To alkylate the samples, add 50 μ l 1 M Iodoacetamide, to give a final concentration of ~200 mM, and incubate at 37 °C for 60 min.
NOTE: If proteomics analysis is not desired, step 1.5 may be skipped.
- Concentrate the denatured glycoprotein onto the filter by centrifuging samples at 14,000 x g for 40 min. Discard flow through.

2. N-linked Glycan Enzymatic Digestion

- Wash the sample with 100 μ l PNGase digest buffer. Concentrate the glycoprotein on the filter at 14,000 x g for 20 min and discard flow through.
 - Repeat the wash and concentrate step twice, for a total of three times, yielding a concentrate in the filter dead volume (~5 μ l). Discard all flow-through.
 - Discard collection vial once washes are complete. Collect all future eluents and washes in a new collection vial.
NOTE: PNGase digest buffer in H₂¹⁸O may be used during the PNGase F digestion step for stable isotope labeling of de-glycosylated asparagine sites, which become chemically deamidated.
- Add 2 μ l of glycerol-free PNGase F (75,000 x units/ml) to filter after transferring to a fresh collection vial. Add 98 μ l of PNGase digest buffer, bringing total volume to 100 μ l, and gently pipette up and down on the filter to mix. Enzymatically digest the glycans under the conditions in steps 2.2.1, 2.2.2, OR 2.2.3.
NOTE: Three methods for the deglycosylation of proteins may be used. For solely glycomics analysis (no proteomics), the long incubation in step 2.2.1 is recommended. For glycomics and proteomics research, steps 2.2.2 and 2.2.3 will produce high quality peptides with minimal non-specific deamidation.
 - Glycomics-only digestion protocol (18hr): Incubate samples at 37 °C for 18 hr to enzymatically cleave all N-glycans.
 - Spike-in digestion protocol (SPI): Incubate samples at 50 °C for 2 hr. Working quickly, remove samples and spike in an additional 2 μ l of glycerol-free PNGase F (75,000 x units/ml). Lightly vortex the samples for 1-2 sec to mix. Incubate the samples at 50 °C for an additional 2 hr.
 - Microwave digestion protocol (MD): Place samples in a microcentrifuge tube floating rack. Float samples in a 1 L beaker filled with 1 L of deionized (DI) water. Place the beaker in the center of a microwave oven (950 W) with a rotating plate. Microwave at 60% power (570 W) for 5 min. To cool, remove samples and hold at room temperature for 2 min. Then microwave for an additional 5 min at 60% power.
NOTE: Microwave power settings will need to be adjusted based on the maximum power output of the microwave. Average power should be held constant between models.

3. Elution of N-glycans

- Elute glycans by centrifuging the sample at 14,000 x g for 20 min at 20 °C.
- Add 100 μ l of PNGase digest buffer to the filter and centrifuge at 14,000 x g for 20 min at 20 °C. Collect wash containing any remaining N-linked glycan, in the same collection vial as the eluent. Repeat twice. Remove filter and place in new collection vial.
- Incubate glycan samples in the -80 °C freezer until frozen (30-60 min). Dry to completion, at room temperature, in vacuum concentrator (4-6 hr).
NOTE: N-glycans may be stored at -20 °C for up to six months prior to derivatization.

4. Protein FASP Digestion

NOTE: If proteomics or deamidation site analysis is not desired, section 4 of the protocol may be skipped.

- Rinse the filter, containing the deamidated peptides, with 400 μ l of 8 M urea buffer. Cap the collection vial and lightly vortex to mix. Concentrate on the filter at 14,000 x g for 15 min. Discard all flow-through.
 - Repeat step 4.1 two additional times, for a total of three urea buffer washes.
- Rinse the filter, containing the deamidated peptides, with 400 μ l of 2 M urea, 10 mM CaCl₂ buffer (trypsin digest buffer). Cap the collection vial and lightly vortex to mix. Concentrate on the filter at 14,000 x g for 15 min. Discard all flow-through.
 - Repeat step 4.1 two additional times, for a total of three trypsin digest buffer washes.
 - Discard collection vial once washes are complete. Collect all future eluents and washes in a new collection vial.
- Add 50 μ l porcine modified trypsin in a 1:50 or 1:5 trypsin: protein (w/w) ratio for discovery or quantitative proteomics experiments, respectively. Cap the collection vial and lightly vortex to mix.
- Incubate samples at 37 °C for 2 hr. Working quickly, remove samples and spike in an additional 50 μ l of trypsin at the 1:50 or 1:5 trypsin: protein ratio. Lightly vortex the samples for 1-2 sec to mix. Incubate the samples at 50 °C for an additional 2 hr.
- Elute the peptides by spinning at 14,000 x g for 15 min.
- Rinse the remaining peptides from the filter with 400 μ l 1% formic acid and 0.001% Zwittergent 3-16 (quench buffer). Elute off the filter by spinning at 14,000 x g for 15 min.
- Quantify the peptide concentration in the samples by Bradford¹⁹ or BCA assay²⁰.
- Incubate peptide samples in the -80 °C freezer until frozen (30-60 min). Dry to completion, at room temperature, in vacuum concentrator (4-6 hr).

NOTE: Dried peptides may be stored at -20 °C for up to three months.
- Resuspend tryptic proteins just prior to liquid chromatography-mass spectrometry (LC-MS) analysis in 2% acetonitrile, 98% H₂O, and 0.1% formic acid (mobile phase A) to desired concentration. Tryptic peptide concentrations from 2.5 μ l plasma range from 100 - 300 ng/ μ l.

5. Derivatization of N-linked Glycans with Hydrophobic Hydrazide Tags

- Reconstitute dried heavy (SIL) or light (NAT) P2GPN reagents in 1 ml of 75:25 MeOH:Acetic Acid (Derivatization solution), for a final concentration of 0.25 mg/ml. Reagents will take up to 10 min to fully solubilize. Extensively vortex to ensure complete solubilization.

Note: In the event this derivatization protocol is used with standard N-glycans, versus purified glycans, the molar ratio of P2GPN: glycan should be approximately (and no less) than 17: 1.
- Tag dried N-glycans with 200 μ l (50 μ g) of SIL or NAT P2GPN reagent. Pipette up and down to resuspend dried glycans. Vortex samples and then spin down samples for ~5 sec on a bench-top centrifuge.
- React the glycans with the reagent for 3 hr at 56 °C.
- Immediately move glycans from the incubator to the vacuum concentrator. Dry to completion at 55 °C (3-5 hr).

NOTE: This step quenches the derivatization reaction; the sample must be completely dry to prevent cross-reactivity in the subsequent steps.

NOTE: Dried, tagged samples may be stored at -20 °C for up to six months.
- Resuspend tagged N-glycans in 25-50 μ l of H₂O just prior to LC-MS analysis. Pipette up and down to ensure N-glycans are fully solubilized.

NOTE: The volume for resuspension is dependent on the starting concentration of glycoprotein, which may be estimated from the starting protein concentration. However, the range will vary per sample type and should be individually optimized.
- Centrifuge samples at 14,000 x g for 5 min. Remove the supernatant, being careful not to let the tip of the pipette touch the bottom of the centrifuge tube.

Note: Excess tag may not be visible to the eye, but will be reduced by centrifugation.
- Combine a pair of NAT and SIL samples, if desired for relative quantification, in a 1:1 ratio for tandem analysis.

6. Ultra-high Pressure Liquid Chromatography and Mass Spectrometry Analysis

NOTE: The LC and MS conditions described for an Easy nLC-1000 and a Q Exactive High Field, respectively, were optimized in-house for proteomics analysis and for glycan analysis²¹. These conditions may be adapted to other ultra-high pressure- or nano- LC systems and other high-resolving power mass spectrometers but may require slight modifications. The use of high resolving power mass spectrometry is necessary for glycan identification and deamidation analysis^{22,23}.

NOTE: Steps 6.1-6.3 can be skipped if an appropriate reverse-phase chromatography or hydrophilic interaction commercial trap and column are used.

- Synthesize a frit in 100 μ M ID capillary according to Meiring *et al.*²⁴ for use as a trap.
- Pack the trap under pressure with 2.6 μ M, 100 Å, C₁₈ packing material and cut to a length of 5 cm.
- Pack a 75 μ M ID emitter column under pressure with 2.6 μ M, 100 Å, C₁₈ packing material and cut to a length of 30 cm.
- Prepare two different LC-MS methods for the proteomics (6.4.1) and glycomics (6.4.2) workflows.

Note: Protein and glycan samples may be run on the same trap/column setup in any order.

 - For protein analysis, inject ~400 ng of protein onto the column in mobile phase A (MPA). Run the sample at 300 nl/min, according to **Table 1**, with a 1 μ l pre-column equilibration (500 bar) and a 5 μ l analytical column equilibration (500 bar). Ionize proteins under the MS conditions provided in **Table 2**.

- For glycan analysis, inject 5 μ l of resuspended, P2GPN tagged NAT/SIL equimolar samples onto the column. Run the sample at 300 nl/min, according to **Table 3**, with a 2 μ l pre-column equilibration (500 bar) and a 5 μ l analytical column equilibration (500 bar). Ionize glycans under the MS conditions provided in **Table 4**.

Time	%MPA	%MPB
0	100	0
5	98	2
105	80	20
135	68	32
136	5	95
151	5	95
152	100	0
167	100	0

Table 1. LC conditions for proteomic analysis. A gradient elution with mobile phase B (MPB, 98% acetonitrile, 2% H₂O, 0.1% formic acid) was performed to elute peptides for shot-gun proteomics, data-dependent-acquisition, MS experiments.

MS ¹ Parameters	
Mass Range (Th)	375-1500
Resolution	120,000
AGC	1 × 10 ⁶
Max Ionization Time	30
S-Lens FR Level	55
Capillary Temp (°C)	300
Spray Voltage	1.75
MS ² Parameters	
Acquisition Type	Top20
Resolution	15,000
AGC	1 × 10 ⁵
Max Ionization Time	30
Underfill Ratio	2%
Isolation Window (Th)	1.4
Charge State Exclusion	+1
Normalized Collision Energy	27
Exclusion Time (s)	20

Table 2: MS conditions for proteomic analysis. The parameters for electrospray ionization, MS¹ acquisition, and MS² acquisition in an orbitrap instrument using higher-energy dissociation (HCD) are given.

Time	%MPA	%MPB
0	95	5
1	70	30
41	60	40
46	37	63
47	10	90
55	10	90
56	95	5
66	95	5

Table 3: LC conditions for glycan analysis. A gradient elution was performed to elute hydrazide tagged N-glycans for MS analysis.

MS ¹ Parameters	
Mass Range (Th)	600-1900
Resolution	60,000
AGC	5 × 10 ⁵
Max Ionization Time	64
S-Lens FR Level	65
Capillary Temp (°C)	325
Spray Voltage	1.75
MS ² Parameters	
Acquisition Type	Top12
Resolution	15,000
AGC	5 × 10 ⁴
Max Ionization Time	100
Underfill Ratio	1%
Isolation Window (Th)	1.4
Fixed First Mass	125
Stepped Normalized Collision Energy	10/20/30
Exclusion Time (sec)	15

Table 4: MS conditions for hydrazide tagged N-glycan analysis. The parameters for electrospray ionization, MS¹ acquisition, and MS² acquisition in an orbitrap instrument using higher-energy dissociation (HCD) are given.

7. Proteomics and Deamidation Analysis

1. Identify proteins/peptides using standard bioinformatics search tools.

Note: The following analysis protocol was designed using the Swiss-Prot/TrEMBL databases in UniprotKB and searching via SEQUEST in the Proteome Discoverer 1.4 software, however, the protocol may be directly translated to any protein search and scoring engine using a GUI software. All commands given below may be accessed in software interfaces through drop-down menus.

1. Create or download an organism appropriate protein sequence database from genomic data or available proteome databases as described by Apweiler *et al.*²⁵

Note: Common proteome databases include Swiss-Prot, TrEMBL, and NCBI, but may be built from in-house data as well.

2. Within the software, choose a database search engine and select parameters according to the quality of the data acquired and the rigorousness of the search desired, as described by Perkins *et al.* for MASCOT²⁶ or Eng *et al.* for SEQUEST²⁷.

1. For high-mass accuracy data, set parameters for the search in the software as follows: max 2 missed cleavages, 5 ppm precursor mass tolerance, and 0.02 Da fragment mass tolerance (for optimized accurate deamidation detection²²) and include the following tryptic peptide modifications: "carbamidomethyl (C)" static modification and "deamidation (N/Q)" and "oxidation (M)" dynamic modifications. If using ¹⁸O digestion labeling, include "deamidation ¹⁸O(1)" as an additional dynamic modification.
2. Search, using the selected engine, the raw peptide data against the protein database (7.1.1).

Note: Mass tolerances should be appropriate for the instrument used and not arbitrarily low.

Note: The search function is completed automatically by the software using various search engine specific algorithms. The percolator algorithm (MASCOT), SEQUEST, or other combinations of algorithms are employed to identify proteins from peptides and to score the validity of the hits; more information on the tools available are covered in a review from Gonzalez-Galarza *et al.*^{28,29}. Depending on the software, additional search parameters may need to be selected according to the discretion of the user.

3. Export the identified peptides for manual curation and further analysis.

2. Manually curate a list of peptides containing an identified deamidation on the asparagine of the N-linked glycosylation motif (N-X-(S/T), where X ≠ P). Cross-validate these sites with known glycosylation motifs from the literature and create two pools of results (validated and theoretical).
3. Use spectral counting³⁰ to compare the percent occupancy of a given glycosylation site by comparing the abundance of deamidated and non-deamidated forms.

8. Glycan Relative Quantitation

Note: The following identification and quantification was completed using the XCalibur software. However, any software that analyzes raw data and automatically integrates chromatographic peaks may be substituted.

1. Generate a theoretical list of glycan compositions from the biologically possible combinations of hexoses, deoxy-hexoses, hexosamines, sialic acids, and other saccharides. For this purpose, a human glycan database has been published by Walker *et al.*¹⁵ and may be used as-is. For theoretical databases, species-specific biological constraints must be imposed on the combinatorial space, and these rules are described by Kronewitter *et al.*³¹.
2. Calculate the glycan monoisotopic masses (M) from the atomic masses for each composition for proton charge states +1-3. Modify the monoisotopic masses to include the addition of the NAT (254.14191 g/mol) or SIL (260.162042 g/mol) P2GPN tag.
3. Open the "Processing Setup" program from the roadmap view in XCalibur. Set up a processing method as exactly described in the Supplemental Material from Walker *et al.*¹⁵ using the list generated in step 8.2 containing the theoretical monoisotopic masses for the [M+H]¹⁺, [M+2H]²⁺, and [M+3H]³⁺ species.

Note: To confirm glycan identity beyond accurate mass, for NAT/SIL duplexed runs, check that NAT and SIL *N*-glycan pairs co-elute with the same retention time. Qualitatively, cross-validate identifications with the MS² spectra, which must contain peaks belonging to the oxonium ion series³².

4. Export the integrated, extracted ion chromatogram (XIC) areas to Excel to obtain the SIL and NAT abundances as exactly described in the Supplemental Material from Walker *et al.*¹⁵
5. In Excel, program the cells in a new column to calculate the correction for the molecular weight overlap by adjusting the SIL abundance according to the equation published by Walker *et al.*¹⁵:

$$(\text{SIL abundance})_{\text{corrected}} = (\text{SIL abundance}) - \left(\frac{A_{\text{NAT}+6}}{A_{\text{NAT}}} \right) \times (\text{NAT abundance})$$

where A is the monoisotopic peak and the theoretical isotope overlap, $\left(\frac{A_{\text{NAT}+6}}{A_{\text{NAT}}} \right)$, may be independently calculated per composition.

6. In a second column, program the cells to normalize the NAT and SIL channels using the equation for the total normalized glycan factor (TGNF), per spectrum, as described by Walker *et al.*¹⁵:

$$\sum_1^N (\text{SIL abundance})_{\text{N,TGNF}} = (\text{SIL abundance}) \times \frac{\sum_1^N (\text{NAT abundance})_N}{\sum_1^N (\text{SIL abundance})_N}$$

where N is the number of *N*-glycans above the limit of quantification.

7. In a new column, program the cells to take the ratio of SIL: NAT glycans (or vice versa) to calculate the fold-change in concentration between the two samples.

Representative Results

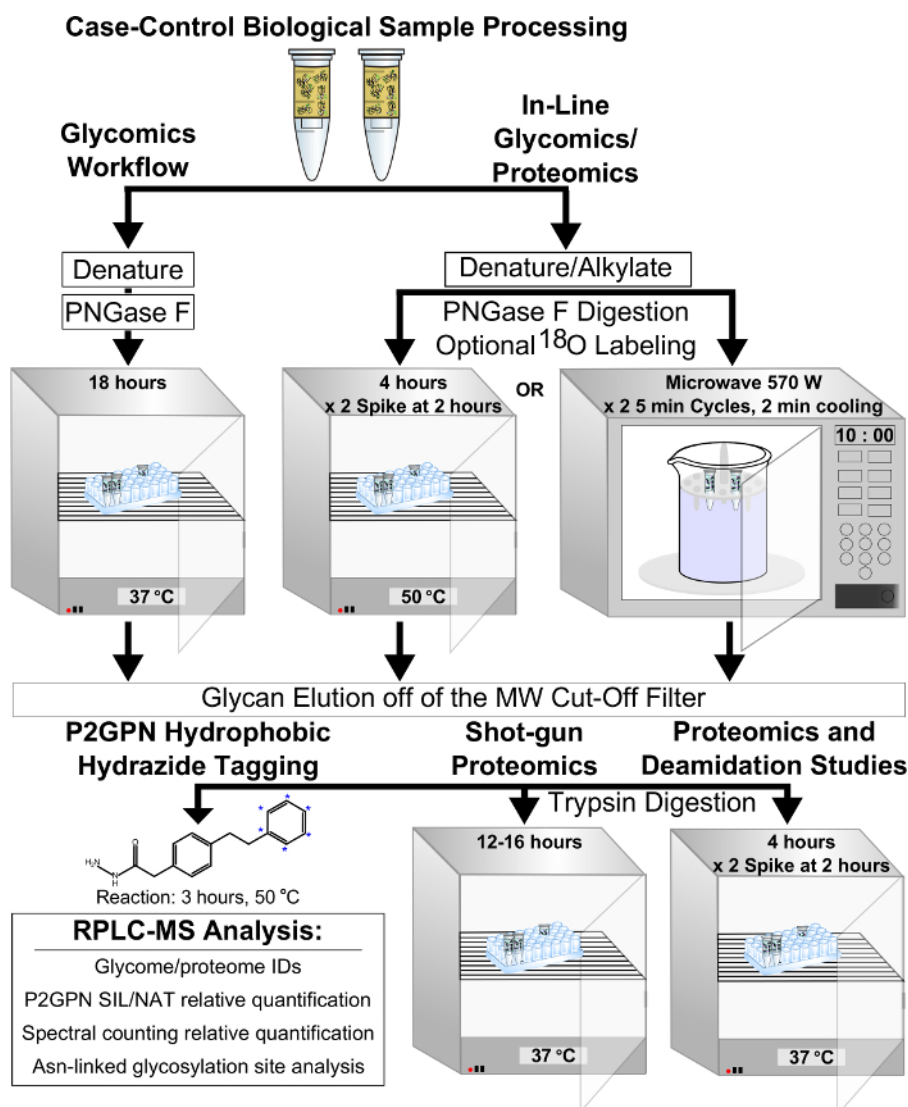


Figure 1: The scheme of the FANGS-P2GPN hydrophobic tagging coupled method (Method A) for combined proteomics and glycomics analysis is given. Steps that differ between glycomics-only processing and tandem glycomics and proteomics analysis, with glycosylation site identification, are highlighted. Please click here to view a larger version of this figure.

The in-line proteomics and glycomics, filter-based P2GPN hydrophobic tagging method (Method A) was validated for identification, quantitation, and molecular weight bias using pooled hen plasma samples (Figure 1). For solely glycomics experiments, inter-comparisons in the abundances of *N*-glycans extracted by Method A to carbograph SPE (gold-standard, Method B) were made (Figure 2). There were no significant differences in the abundances of glycans between the two methods. The intra-variability was also assessed by quantifying the SIL: NAT ratio of glycans extracted by the same method. The log₂-distributions of both methods were Gaussian, centered on zero, and not significantly different (Figure 3A-B). The molecular weight ranges between the two protocols completely overlapped, suggesting that the filter did not discriminate *N*-glycans based on molecular weight range and hydrophilicity (Figure 4).

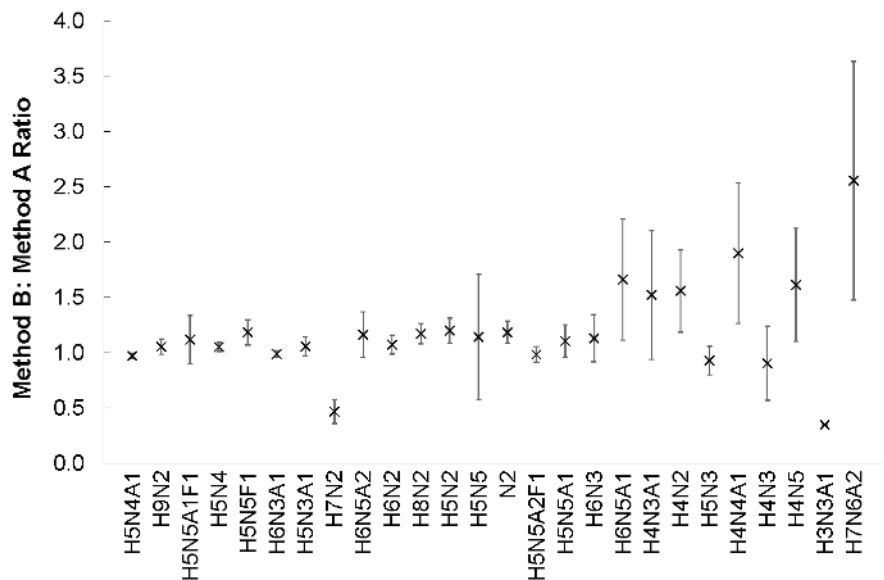


Figure 2: An equimolar mixture of NAT and SIL *N*-glycans extracted by Method A or Method B was prepared from 2.5 μ l of hen plasma ($N = 4$). Samples were analyzed by UPLC-MS according to the recommended parameters suggested in section 6. The abundances for each glycan, in each NAT/SIL channel, were calculated by integrating the area under the extracted ion chromatogram (MMA = 3 ppm), correcting for the molecular weight overlap, and adjusting by the TGNF. These ratios are shown with their standard errors. The abundances of Method B: Method A *N*-glycans were not significantly different ($p > 0.05$). [Please click here to view a larger version of this figure.](#)

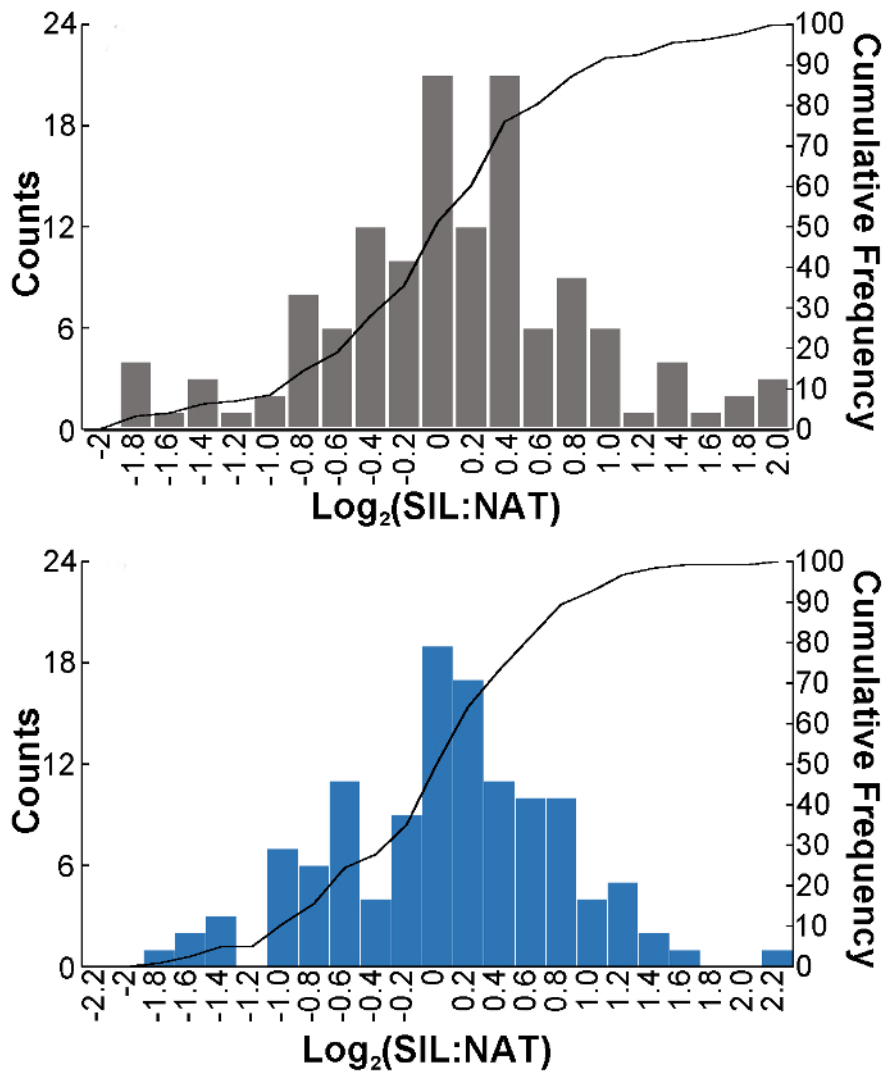


Figure 3: The intra-variability in the (A) Method A (N = 133) or (B) Method B (N = 123) strategies was compared. NAT and SIL equimolar mixtures of *N*-glycans, from the same extraction scheme, were analyzed over three technical replicates. The log₂-distributions were centered on zero and were not significantly different between the two workflows. [Please click here to view a larger version of this figure.](#)

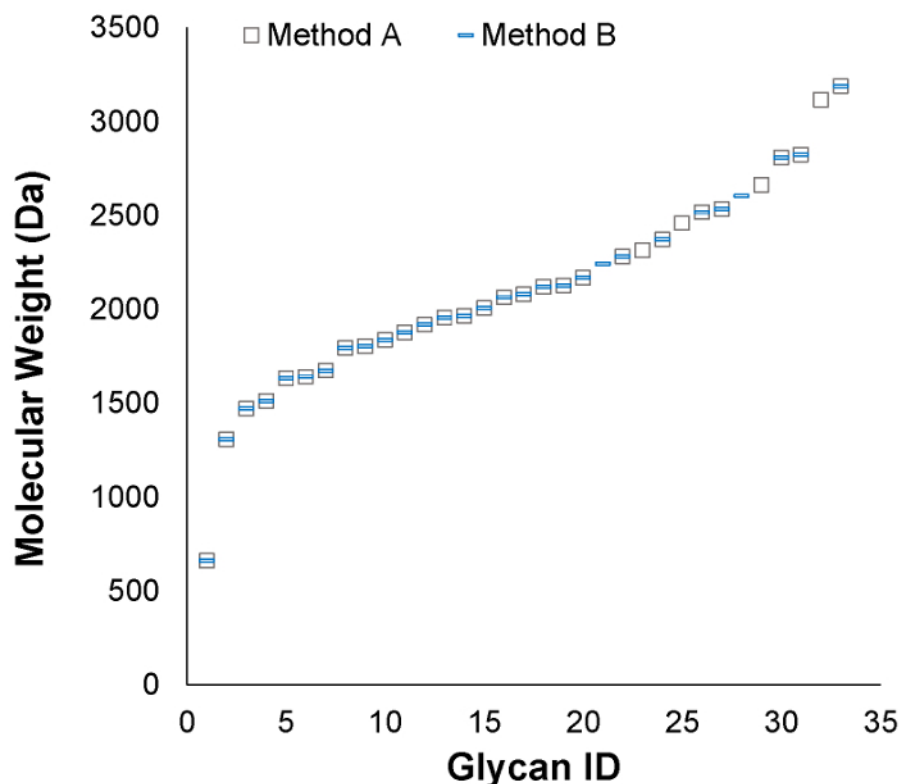


Figure 4: The molecular weight ranges of glycans from each strategy were compared. The two workflows yielded glycans spanning the same molecular weight range. *N*-glycans detected in one protocol versus the other fell just above the limit of detection (1×10^5 abundance) and do not reflect systematic bias. [Please click here to view a larger version of this figure.](#)

Retention of the deglycosylated proteins by the molecular weight filter enabled in-line proteome wide analysis and glycosylation site identification. Multiple protocols for the combined purification of glycans and proteins were compared to a traditional FASP preparation without deglycosylation (**Table 5**). Our typical 18 hr filter based PNGase F digestion, followed by FASP trypsin digest (Std protocol) resulted in significant levels of non-specific deamidation, which can interfere with the identification of glycosites. Therefore, a method utilizing a shorter incubation time at elevated temperature (50 °C) and a PNGase F spike-in step (SPI protocol) was explored as an alternative, along with a microwave digestion protocol (MD protocol), to minimize non-specific deamidation. The glycans observed in the new digestion methods were not significantly different in compositions or abundances from the standard 18 hr, 37 °C filter PNGase F digest (**Figure 5**). Combined with a short trypsin incubation, non-specific deamidation was significantly reduced, with a false positive rate for glycosites <5% (**Table 5**).

	Proteins		Peptides		Deamidated Peptides		Glycosites		Glycoproteins	
	+	-	+	-	+	-	+	-	+	-
FASP		249		3083		795		5		5
18hr	217	304	6013	5086	1029	733	257	24	112	18
MD	270	266	5190	5125	465	455	254	8	102	7
SPI	281	288	4729	4482	602	573	232	10	145	8

Table 5: Comparisons of proteomic data from a standard trypsin digest versus the in-line FANGS-P2GPN tagging method were made. Preparations were performed with (+) and without (-) PNGase F to determine background rates of non-specific deamidation and estimate glycosite false positive rates. Proteins were identified with 1% false discovery rate (FDR); peptides were filtered based on "high" peptide confidence in Proteome Discoverer 1.4 ($q < 0.01$); glycosites were identified based on unique sequences containing deamidation of the conserved glycosylation motif (N-X-S/T); and glycoproteins were defined as identified proteins containing at least one identified glycosite.

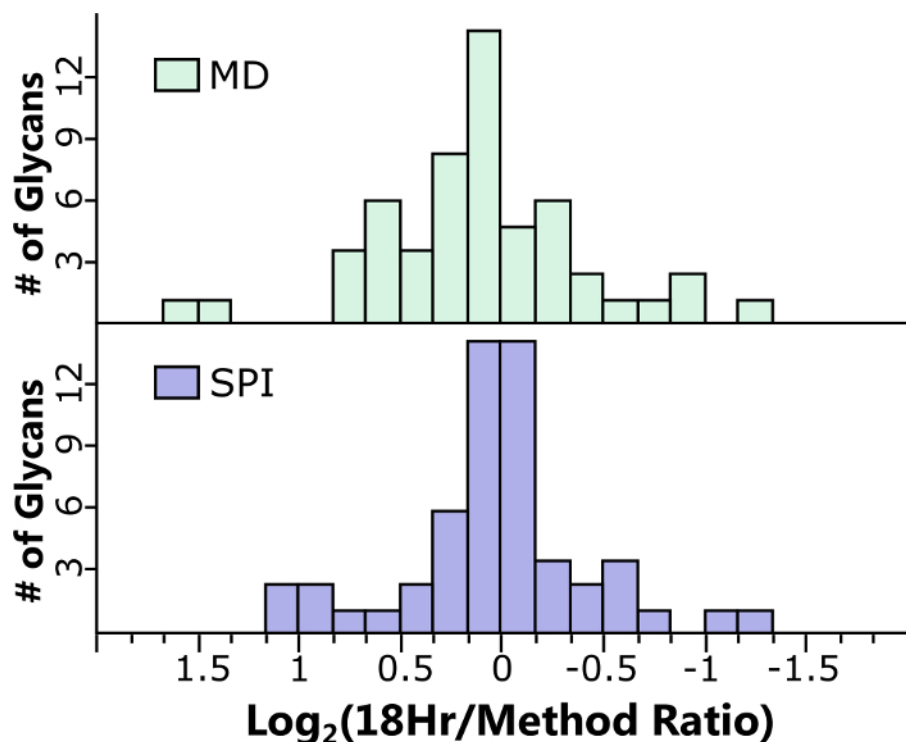


Figure 5: Shortened protocols for glycans were developed to minimize deamidation in the samples and compared to the 18hr FANGS PNGase F digest. The average differences in abundances were 10% and 5% for the MD (2.2.3) and SPI (2.2.2) protocols, respectively. Of the 48 glycans identified, 90% displayed less than 1.5-fold variation. [Please click here to view a larger version of this figure.](#)

Discussion

High-throughput quantitative methods are needed to facilitate routine glycan analysis. For the last thirty years, glycomics analysis has been limited to a subset of research groups, despite its importance in disease, clinical applications, and pharmaceuticals. The FANGS-P2GPN purification and tagging method for glycomics and proteomics performs the same analysis on a single aliquot of sample, reducing the cost of supplies and the amount of material needed (particularly important in human and mouse studies). Furthermore, efforts to minimize variability in preparations are critically important, as every additional step contributes to error, potentially masking important but low-abundant changes in case-control studies. Coupling of FANGS to hydrophobic hydrazide tagging allows protein and glycan samples to be run on the same RPLC column, enhances glycan ionization, provides for relative quantification, and can be quantitatively applied to plasma.

For *N*-glycan analysis, it is critical to use the suggested level of PNGase F to achieve full de-glycosylation. Though glycans are solvent exposed, denaturation of proteins and excess enzyme help ensure efficient and complete cleavage. For accurate quantitation of the glycans, it is necessary to ensure that they are completely dried after derivatization to quench the reaction and prevent cross-reactions when mixing the NAT and SIL species. Finally, when extending the workflow to glycosite analysis, timing of the steps is critical to minimize non-specific deamidation. The modified protocols provided for combined glycomics and proteomics analysis work consistently when performed accordingly.

The workflow achieves accurate relative quantitation of *N*-glycans from plasma compared to the gold-standard, SPE method. There is no apparent bias in the types of glycans extracted in terms of molecular weight, hydrophilicity, and compositional structure. Though we have not explored the qualitative analysis of *O*-linked glycans, we expect that FANGS could accommodate the addition of a β -elimination step post-PNGase F digestion of *N*-linked glycans. However, procedures would require significant modification for reagent cleanup prior to mass spectrometry, and peptide analysis will be significantly impacted. For proteomics, the same depth of proteome coverage is achieved compared to traditional FASP methods. Importantly, methods achieve a minimal false discovery rate for *N*-glycan deamidation. While the method is compatible with ^{18}O labeling of Asn during the PNGase digestion step^{22,23}, the low glycosylation site false discovery rate suggests that it may not be necessary, further reducing costs and complexity.

The proteome is not enriched for glycoproteins in this method, which has both advantages and disadvantages. Certain low abundant glycoproteins may not be detected in the analysis. However, the occupancy of glycosylated sites per protein, can be compared between biological samples. Additionally, the error and bias introduced from lectin affinity purification or chemical enrichment is eliminated. In conclusion, coupling of FANGS to the individuality normalization when labeling with glycan hydrazide tags strategy results in a simplified, quantitative, high-throughput method for the tandem analysis of the glycome and proteome with great potential for application in clinical case-control studies.

Disclosures

We have a collaborative relationship with Cambridge Isotope Laboratories (CIL) to disseminate the P2GPN reagent and technology to the broader scientific community in the form of a product (GTK-1000). However, we do not receive any revenue based on the sale of this product.

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