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Title:

Interlaboratory Study on Differential Analysis of Protein Glycosylation by Mass Spectrometry: the ABRF Glycoprotein Research Multi-Institutional Study 2012

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Abbreviations

ABC	Ammonium Bicarbonate
Acc	Accuracy
AHC	Agglomerative Hierarchical Clustering
Alk	Alkylation
ArgC	Endoproteinase ArgC
BABAC	2-bromoethylammonium Bromide (S-aminoethylcystein)
BU	Bottom Up
CE,	Capillary Electrophoresis
HPAED	High Performance Anion Exchange Chromatography
Chrom	Chromatography
Chym	Chymotrypsin
CID	Collisionally Induced dissociation
dHex	deoxyhexose
IAA	Iodoacetamide
Enz	Enzyme
ESI	Electrospray ionization
ETD	Electron Transfer Dissociation

Gdn	Guanidine
HexNAc	*N*-acetylhexosamine
HCD	Higher energy Collision Dissociation
Hex	Hexose
LysC	Endoproteinase LysC
Lysyl Endo	Lysyl Endoproteinase
Mercaptoethanol	ME
MTS	Methanethiosulfonate
Neu5Ac	N-acetyl neuraminic
Neu5Gc	N-glycolyl neuraminic
MS	Mass spectrometry
OV	overnight
Permet	Permethylated
PGC	Porous Graphitic Carbon
PLRP-S,	Polymeric Reversed Phase
Phos	Phosphate
Qty	Quantity
Res	Resolution

Red	Reduction
TCEP	Tris(2-carboxyethyl)phosphine
TEAB,	Tetraethyl Ammonium Bicarbonate
TPCK	N-Tosyl-L-phenylalanine chloromethyl
Tryp	Trypsin
SID	Surface-Induced Dissociation

Summary

One of the principal goals of glycoprotein research is to correlate glycan structure and function. Such correlation is necessary to understand the mechanisms whereby glycoprotein structure elaborates the functions of myriad proteins. Accurate comparison of glycoforms and quantification of glycosites is an essential step in this direction. Mass spectrometry has emerged as a powerful analytical technique in the field of glycoprotein characterization. Its sensitivity, high dynamic range, and mass accuracy provide both quantitative and sequence/structural information. As part of the 2012 ABRF Glycoprotein Research Group (gPRG) study, we explored the use of mass spectrometry and ancillary methodologies to characterize the glycoforms of two sources of human prostate specific antigen (PSA). PSA is used as a tumor marker for prostate cancer, with increasing blood levels used to distinguish between normal and cancer states. The glycans on PSA are believed to be biantennary *N*-linked and it has been observed that prostate cancer tissues and cell lines, contain more antennae than the benign form. Thus, the ability to quantify differences in glycosylation associated with cancer has the potential to positively impact use of PSA as a biomarker. We studied standard peptide based proteomics/glycomics methodologies including LC-MS/MS for peptide/glycopeptide sequencing and label-free approaches for differential quantification. We performed an interlaboratory study to determine the ability of different laboratories to correctly characterize the differences in glycoforms between two different sources using mass spectrometry methods. We used clustering analysis and ancillary statistical data treatment on the data sets submitted by participating laboratories to obtain a consensus of the glycoforms and abundances. The results demonstrate the relative strengths and weaknesses of top-down glycoproteomics, bottom-up glycoproteomics, and glycomics methods, respectively.

Introduction

The fact that most proteins are glycosylated underlies the critical roles played by glycosylation during evolution; glycoconjugate expression is a key mechanism whereby organisms maintain fitness in response to evolutionary pressures (1). All living cells are coated with glycoconjugates, through which they interact with their environment. Glycans consist of combinations of epitopes built on common core structures (2). These epitopes are recognized by carbohydrate binding lectin domains present in numerous protein families. Thus, glycosylation serves to modulate the adhesive properties of a glycoprotein. In this way, the function of a glycan arises through the protein(s) to which it binds.

The attachment of *N*-glycans to proteins in the endoplasmic reticulum is part of the calnexin-calreticulin protein folding quality control pathway. Glycans may subsequently be elaborated in the Golgi apparatus to acquire complex architecture resulting from the actions of a series of biosynthetic enzymes. The mature glycan structures are heterogeneous, a reflection of the fact that the biosynthetic events do not go to completion. It appears that function arises in the context of heterogeneous glycans built on common cores. This heterogeneity of *N*-glycans has biological impact through elaboration of the physico-chemical properties and biological functions of proteins.

Protein *N*-glycosylation occurs primarily on NXT/NXS consensus sites, X should not be P. Although a given protein may have several such consensus sites, typically not all are occupied. Partial occupation of a consensus site in a population of protein molecules is also possible. The structures of glycans on a given glycoprotein molecule differ depending on the glycosylation site. The *N*-glycan structures are generally classified into 3 different categories: high mannose, complex and hybrid types and are characterized by a common chitobiose core ($Man_3GlcNAc_2$). Thus, glycan structure at a given site impacts protein function through the lectin-glycan binding interactions it enables. As a result, it is necessary to develop analytics capable of confident mapping of the *N*-glycan structures present on a

given asparagine residue on a given glycoprotein. The range of *N*-glycans expressed becomes altered during development of cancer (3-5). Specifically, tumor cells display increased β1-6 branching of *N*-glycans due to upregulation of GlcNAc transferase V (6-8). In addition, the change of the amount and the linkage of sialic acids (9-12), and the expression of *N*-glycolylneuraminic acid in human tumors (13-14) have been described, but requires structural detail in order to improve understanding from a mechanistic and pathological point-of-view.

The goal of glyco analytics is to determine glycoprotein structures that represent those giving rise to biological function in a given context. Accuracy has been the enabling technology of proteomics and glycomics. Profiling produces information on the composition of biomolecules. The tandem mass spectrometry dimension confirms the composition (15-16). The high sensitivity, dynamic range, and mass accuracy of tandem mass spectrometry data may provide both quantitative and sequence/structural information that, in principle, enable systems-wide "omics" experimentation for glycoproteins (17-18).

In recent years, a Human Proteome Organization (HUPO) study compared methods for profiling glycoprotein *N*-glycans (19). This study demonstrated the effectiveness of matrix-assisted laser desorption (MALDI)-time-of-flight (TOF) MS for profiling of permethylated glycans and of Electropspay ionization LC-MS for profiling of native and reductively aminated glycans. Most of the glycopeptide data submitted for this study were qualitative in nature. A second HUPO study focused on methods for profiling of *O*-glycosylation. Two approaches were particularly effective: the direct MS analysis of permethylated reduced glycans and the analysis of native reduced glycans in negative mode by LC-MS (19-20). This study was semi-quantitative in nature.

The focus of the 2012 gPRG ABRF study was on evaluating methods for site-specific glycoprotein glycosylation profiling (18, 21-23). In order to determine relative quantities of specific glycans occupying

a given site, it is necessary to detect the glycopeptides or glycoprotein directly. This requirement is the crux of the analytical challenge. Glycosylation decreases the peptide hydrophobicity and thus the ionization efficiency for glycopeptides. The heterogeneous nature of glycosylation complicates the task of assigning observed masses to compositions. A peptide containing an *N*-glycosylation consensus sequence is expected to be modified with a series of glycan compositions. Thus, it may be necessary to use tandem MS in order to assign unambiguously the composition of a glycan that modifies a given peptide.

The goal of this study was to determine the ability of the field to conduct comparative analysis of glycoprotein glycosylation using PSA as the target. We selected human prostate specific antigen (PSA) as the target molecule for the present study for two reasons. First, PSA is a low molecular weight glycoprotein (MW ~30,000 Da) characterized by a single site of *N*-glycosylation at Asn-69. This single glycosylation site permitted participants to use either bottom-up, PNGase F or top-down glycosylation profiling. Second, there is significant biological interest in PSA glycosylation structure: PSA is used as a screening biomarker for prostate cancer. PSA is secreted as a proenzyme into the lumen of the prostate gland. In cases of prostate disease, the basement membrane can become disrupted, resulting in PSA access to the peripheral circulation (24); however, the correlation between concentration of PSA and cancer is not absolute. If the concentration of PSA in blood plasma is in the range of 2.5-10 ng/ml, a man has 25% chance of prostate cancer vs 75% chance of no disease, prostatitis or benign prostatic hyperplasia. If the concentration of PSA in the plasma increases to higher than 10 ng/ml, the likelihood of cancer increases to >60%. Age, race, family history of prostate cancer, PSA level, PSA velocity, digital rectal examination result, and previous prostate biopsy are factors that can influence the probability of cancer. There is a strong need to improve the specificity of prostate cancer detection in order to minimize harm to patients through unnecessary surgical treatment. Researchers have tried different approaches for improving this specificity. In the blood, PSA may be free or complexed with alpha-1-

antichymotrypsin or alpha macroglobulin. The ratio of PSA free vs. complexed may serve as an indication of prostate cancer (25). The detection of cancer-specific Isoforms of PSA may permit discrimination between benign and malignant cases (26-27). Another promising approach is related to the glycosylation profile of PSA. There is a great deal of interest in exploiting the alteration of protein glycoforms that may occur with cancer development in order to improve biomarker performance (28-30).

The rationale of the study was to determine the ability of the international glycoproteomics community to compare *N*-glycosylation between two different sources of PSA by mass spectrometry. For practical reasons, including the need to obtain samples in adequate quantity, purity and cost, we chose a commercially available source of PSA. Two different forms of PSA with different glycosylation profile were obtained by the commercial vendor from bulk purification of seminal fluid. The PSA samples were characterized by the study organizers to demonstrate their suitability. These preliminary data were not shared with the participants. In total, 35 samples of PSA and PSA high isoform were sent to laboratories around the world and 25 data sets were returned. We present the study results, an a global overview of the approaches and methodologies used for differential characterization, and highlight challenges faced by researchers in this area. We used statistical methods and comparative analysis to derive a consensus of the interlaboratory data with the ultimate goal to compare the glycoforms both qualitatively and quantitatively. We evaluated which sample preparation, separation and analysis methods produced the most consistent results. We then built a consensus data set for the two sources of PSA and used it to show which glycan compositions differ significantly in abundances between the two sources. The results demonstrate the challenges to achieving reproducible results using bottom-up analysis of proteolytic glycopeptides.

Methods

Guidelines for the 2012 ABRF gPRG Study (**Supp. Figure 1**)

A description of the glycoproteins and a brief background on the study was sent to each participant along with the samples. We highlighted different approaches, top-down, bottom-up and analysis of released glycans that the participants might explore in their approach, based upon preliminary analysis of the samples in our laboratory. The term "top-down" includes intact protein mass profiling and intact protein tandem MS. We suggested that participants use bottom-up LC-MS/MS and label free quantification on glycopeptides for both qualitative and differential quantitative analysis. We also asked participants to consider carefully the choice of enzyme, cleanup, chromatography, mass spectrometry (high and low resolution and mass accuracy) and tandem mass spectrometry methods.

Samples

PSA and PSA high isoform samples were obtained as a gift from Lee Biosolutions (St. Louis, MO). PSA and PSA High Isoelectric Point Isoform (PSA high isoform) were purified from seminal fluid in lot-matched batches. PSA high isoform was obtained through a proprietary process at Lee Biosolutions used to bulk purify proteins dependent upon isoelectric point. Each participant received 100 µg of PSA (lot number M02015) and 20 µg PSA high isoform (lot number M15097) in the same buffer. The proteins were stored in 0.05M phosphate buffer, pH 7.5, containing 0.15M NaCl, 0.09% NaN_3 at 4^{o}C. The samples were shipped using cold packs in a liquid state.

Participating Laboratories

In total, 35 PSA sample sets were sent to laboratories in North America (20), Europe (12), Australia (1), Japan (1), and China (1). After analysis, 24 laboratories sent back 26 data sets. Laboratories 2 and 9 completed 2 different data sets (designated a and b), one using bottom-up glycopeptide analysis and the other for glycans released using PNGase F. The survey results of laboratories 11, 15, 17 and 24 were not

complete and consequently were not included in the final differential statistical study; however, their results were used to confirm observations made by the other participants.

Methodology

The analytical approaches that were used by the different participating laboratories are summarized in **Table 1.** They included analysis of glycoprotein - top-down approach, analysis of glycopeptides - bottom-up approach; and analysis of released glycans - PNGase F approach. Traditional LC MS/MS for peptide sequencing and label free differential quantification as suggested in the participant guide was used by 17 participants. Four participants performed top-down analysis. Deglycosylation by PNGase F and analysis of the released *N*-glycans was performed by 5 participants. The analytical protocols used by each of the laboratories, including preparation of samples, enzymatic digestion conditions and sample cleanup, are provided in **Supp. Figure 2.**

Bottom-up methods

Glycopeptide analysis and label free quantification were used to profile the *N*-glycans by 17 of 24 participating laboratories. Trypsin was the most popular choice of enzyme (laboratories 1-10). Other enzymes used were: chymotrypsin (laboratories 11, 12)(31), chymotrypsin/trypsin (laboratory 13), endoproteinase Arg-C (laboratory 14), endoproteinase Arg-C/trypsin (laboratory 15), and endoproteinase Lys-C (laboratory 16) and lysyl endoproteinase (laboratory 17). LC-MS with reversed phase C_{18} chromatography was used by 11 laboratories (1-8 and 11-15). Other approaches included: capillary electrophoresis (CE) (laboratory 10), porous graphitized carbon (PGC) chromatography (laboratory 9) and reversed phase C_8 chromatography (laboratory 16). Laboratories 1 and 2 enriched the products obtained from the trypsin digestion with hydrophilic interaction chromatography. Laboratory 17, based upon preliminary MALDI analysis, used a C_{30} reversed phase cleanup approach to separate the

glycopeptides from peptides. Three laboratories (2, 9 and 17) used low resolution mass spectrometry (fwhm) (R=5,000) while 7 (1, 6, 12, 14, 21, 22 and 23) used instruments with resolution between R=10,000 and R=20,000. Resolution greater than R=20,000 was used by the other participants. The method of ionization used by all except laboratory 17 (MALDI) was electrospray. More than 80% of the participants acquired tandem MS data. Two laboratories (laboratory 8 and 18) used electron transfer dissociation (ETD) to identify the site of glycosylation while the other groups used collision-induced dissociation (CID) or higher energy C-trap dissociation (HCD) or a combination of CID/HCD. Laboratories 9, 2 and laboratories 18, 19 used the bottom-up approach in parallel with either PNGase F or top-down methods. Laboratories 2 and 9 furnished a complete data set for both experiments. We note that the results indicating serious analytical problems (laboratories 11, 15 and 17) were not included in the final data analysis. Laboratory 15 did not detect any sialylated compounds, laboratory 11 enriched the chymotrypsin obtained glycopeptides following digestion using a sialic acid capture-and-release protocol (31), furnishing a partial *N*-glycan profile, and laboratory 17 did not present any data corresponding to the glycoforms of PSA.

Top-down MS and tandem MS

Intact protein mass profile measurements and top-down MS/MS were performed by laboratories 18(b), 19(b), 20 and 21, each of which opted for a different type of separation prior to MS: PLRP-S chromatography, reversed phase monolithic chromatography, reversed phase C_8 and CE. Laboratories 18(b) reduced and alkylated the proteins prior to MS. Tandem mass spectrometry approaches involved with the top-down analysis were ETD and HCD for laboratory 18(b). Resolution higher than R=240,000 and a mass accuracy better than 10 ppm were obtained. Laboratory 19 chose a 2 step strategy to establish the *N*-glycan profiles. First a bottom-up approach using Arg-C digestion allowed determining the *N*-glycan compositions. The resulting *N*-glycan compositions were used to facilitate the

interpretation of the MS profile of the intact protein isoforms, and quantification was established on this intact protein profile. The resolution of the mass spectrometer used by this group was R=40,000 with a mass accuracy of approximately 10 ppm. Laboratories 19(b), 20 and 21 did not acquire any top-down tandem MS data. The deconvoluted mass spectra allowed interpretation of the *N*-glycan compositions by matching the mass differences with possible glycan compositions. Prior to the mass spectrometry analysis, laboratory 21 separated the PSA sialylated glycoforms using CE. Laboratory 19(b) fractionated the glycoprotein using a RP-H4 column, and laboratory 20 fractionated the glycoprotein using C_8 reversed-phase chromatography.

Deglycosylation by PNGase F and analysis of released *N*-glycans

Release of glycans using PNGase F followed by characterization in MS was performed by 5 laboratories. Laboratories 9(b), 2(b)(32) and 22 detected the released glycans by PGC LC-MS. Laboratory 21 used MALDI TOF MS to detect permethylated *N*-glycans. Laboratories 9 and 2 obtained CID tandem mass spectrometry data. The mass resolution used by the participating laboratories ranged from R=2,000 to R=10,000. Laboratory 24 identified released *N*-glycans using high pH anion exchange chromatography with a pulsed amperometric detector. The glycans of PSA and PSA high isoform were identified by comparing the elution time of those compounds with those of standards. Some glycans present between 20 and 40% of relative intensity were not characterized and remained unknown. This *N*-glycan profile therefore presented too many unidentified glycans and was thus not included in the statistical analysis of the interlaboratory data.

Interpretation of tandem MS data

More than 75% of the participating laboratories acquired tandem MS data to propose the *N*-glycan profiles of PSA and PSA high isoform. While MS data might allow one to propose a composition based

upon the mass accuracy, mass differences and knowledge of the compounds studied (glycans, glycopeptides, glycoproteins), tandem MS data confirms the assignment. The majority of laboratories analyzed the tandem data manually, clearly indicating the lack of informatics tools necessary for their interpretation, **Supp. Figure 3**. Laboratories reported using the following software for bottom-up data: FindPept (33) (laboratory 2(a), Glycopep (34) (laboratory 3), SimGlycan (35) (laboratory 8), and GlyPID (36) (laboratory 6). One laboratory used the Prosight PC 3.0 (37-38) software for top-down data. Laboratory 19(a) used a combination of processing of bottom-up data using ProteinScape and Glycoquest (Bruker Daltonics) to interpret the glycopeptides obtained from an Arg C digestion. After interpretation of the glycans, they reported the global interpretation from top-down data. Laboratories 20 and 21 did not acquire tandem MS data and presented their results solely based upon the MS profile. Laboratories 2 and 9 performed a bottom-up approach, reported as 2(a) and 9(a), and analysis of glycans released using PNGase F digestion, reported as 2(b) and 9(b).

Data Analysis

Each participating laboratory furnished a list of *N*-glycan compositions with their corresponding relative intensities for PSA and PSA high isoform. The lists ranged from 8 to 58 glycan compositions. Prior to processing via statistical treatment, all compounds with intensity lower than 0.1% and/or were observed by only one laboratory, were removed from the data set. Combining data from the 22 complete data sets, a total of 60 compositions passed this filter.

Statistical Data Analysis

The PSA samples represented the type of analytical challenge one might expect to characterize within a core laboratory. While example protocols were suggested, the participants chose the methods used. The consequence was that it was necessary to use statistics appropriate for the variety of methods used

and the number of participant data sets. To model the data across participating laboratories, primary statistical treatment using agglomerative hierarchical clustering (AHC) was performed. AHC (39-40) is one of the most common statistical tools used to define the degree of similarity/dissimilarity between objects and groups. It allows for iterative grouping or segmentation of objects, in this case intensities of *N*-glycan compositions from PSA and PSA high isoform obtained from different laboratories. Statistical analyses using AHC and manual confirmation of the clustering results were performed using R scripts and spreadsheets (41). Clustering was then performed to group the results from the participants.

Another goal of the study was to perform a comparison of the reliability and reproducibility of the methods employed by the laboratories in this study. We performed permutation tests to attempt to establish a ranking in the standard deviation of measured abundances, averaged across all glycans for each methodology (top-down, bottom-up, and PNGase F release). We calculated this average standard deviation for the true data from 22 laboratories, and then for 10,000 permutations of methodology assignments and compared differences in our actual average standard deviations to those in our resampled data. AHC was then applied to the data to determine which *N*-glycans differed between the PSA and PSA high isoform and in which proportion. We then applied the Wilcoxon signed-rank test (W test) to the data. The W-test is a no*n*-parametric test that evaluates the difference in the mean values of the two sample sets (PSA and PSA high isoform) and assigns for each glycan composition a p-value, as recommended by Cairns (42) and Dakna (43). The threshold of significance for the p-value was established at 0.0008, which is the standard 0.05 confidence level adjusted for 60 tests conducted using a Bonferroni correction. The data were then plotted as a heat map and a second application of AHC was performed across the *N*-glycans and the differences in intensity between PSA and PSA high isoforms.

Results-Discussion

The study results were broken down into three aspects: sample integrity, qualitative analysis and

differential quantitative analysis. Preliminary analysis by the study organizers demonstrated that the PSA and PSA high isoform samples were of sufficient integrity for the interlaboratory study. Qualitative analysis was primarily concerned with determining the site of *N*-glycosylation and the description of the glycans contained in PSA and PSA high isoform. Correlation of the results obtained from different laboratories was then performed in order to rank the experimental approaches. Finally, we measured the degree of consistency in the differential quantification of glycans in order to determine the best consensus set of results.

Sample Integrity

Prior to shipment, the purity of the samples was assessed using LC-MS-based proteomics and SDS-PAGE, **Suppl. Figure 1**. As the samples were shipped with cold packs to participating laboratories we took note of the transport time for each sample, which varied from overnight to more than 7 days. The question of degradation of the proteins was thus a concern (32). We addressed this problem by measuring the results of two laboratories that received the samples within different time frames: 24 hours (laboratory 23), and more than 7 days (laboratory 9). Laboratory 9 profiled the glycans of PSA by immobilizing PSA on PVDF membrane, digesting with PNGase F, reducting the released *N*-glycans, and cleanup by methanol precipitation. Laboratory 22 deglycosylated the PSA using PNGase F in solution, followed by permethylation of the glycans and subsequent MS analysis. The glycans profiles of PSA and PSA high isoform obtained by these 2 laboratories were similar and indicated that the proteins had not degraded significantly during transportation (**Supp. Figure 4)**.

Qualitative Analysis of Glycan Isoforms on PSA and PSA high isoform

Site Determination

Almost all laboratories using bottom-up or top-down methodology correctly assigned the *N*-

glycosylation site at position Asn-69 for PSA and PSA high isoform. Laboratories (laboratory 15, 17, 20, 21) did not perform MS2 experiments to identify the site of glycosylation.

Four laboratories reported additional glycosylation sites. 2 Laboratories 18(a) and 19 (a) reported a *N*-glycosylation site at Asn-78, presumed to be due to a mutation of aspartic acid to asparagine for the PSA, described as a high mannose type with a main composition of $Hex_5HexNAc_2$. This mutated form of PSA is reported by laboratory 19(a) as a trace contaminant of the PSA sample. We noted that the identified peptide sequence SFPHPLY**N**MSLLK is homologous to a peptide of kallikrein 2 (KLK2) and the sequence homology between KLK2 and PSA (KLK3) is approximately 77%. KLK2 plays a role in proteolytic activation of PSA (44) and there was a possibility that KLK2 was co-isolated with PSA. KLK2 was not detected in our proteomics data. The results issued from the bottom-up approach of the laboratory 18 (a) remove the ambiguity of the presence of KLK2 as a contaminant. The identified peptide sequence reported by the laboratory 18(a) was SVILLGRHSLFHPEDTGQVFQ*VSHSFPHPLY**N**MSLLK* and not the identified sequence NSQVWLGRHNLFEPEDTGQRVPV*SHSFPHPLY**N**MSLLK* issued from KLK2.

Laboratory 11 performed enrichment of the glycopeptides prior to MS analysis by using a sialic acid capture-and-release procedure; the results were consistent with the presence of an *O*-glycosylation site for PSA high isoform only. The *O*-glycosylation site was reported on the amino acid Ser-23 based on tandem MS data. A Mascot search identified the peptide sequence LIL**S**RIVGGW carrying the *O*-glycan composition HexNAcHexNeuAc. Laboratory 24 used an indirect method to identify potential *O*-glycosylation. The amounts of glucosamine (GlcN) and galactosamine (GalN) were determined using monosaccharide analysis of acid hydrolyzed samples of both the intact PSA samples. There was a slight increase in the amount of GalN in PSA high isoform when the entire protein was hydrolyzed compared to PSA, suggesting an increased amount of *O*-glycosylation in the PSA high isoform. In parallel, the *N*-glycans of both proteins were released by PNGase F and isolated. GalN and GlcN amounts in the *N*-

glycans were measured and observed to be constant for the released *N*-glycans; however, given that other proteins were present in the samples, it cannot be determined conclusively that the unaccounted for GalN abundance was due to *O*-glycosylation of PSA.

N- Glycans of PSA and PSA high isoform

The 22 complete data sets identified 142 glycoforms for PSA and PSA high isoform together. Each glycoform that was observed by only one laboratory or with intensity lower than 0.1% was eliminated. A total of 61 glycoforms passed this initial filter for PSA and PSA high isoform, reducing the initial data set by more than 57%. This reduction in data, according to method used, was: top-down 27% of the non-significant compounds, bottom-up 16% and PNGase release 14%.

The *N*-glycans of PSA and PSA high isoform with a relative intensity >0.1% and detected by more than one laboratory were then classified into three sub-groups to facilitate data interpretation, **Table 2**:

1. Major group *N*-glycans: 7 *N*-glycans are observed by more than 65% of the participants.
2. Intermediate group *N*-glycans: 11 *N*-glycans were observed by 30-65% of the participants.
3. Minor group *N*-glycans: 43 *N*-glycans were observed by less than 30% of the participants.

The number of observed *N*-glycans correlated inversely to intensity. Qualitatively, 37% of the *N*-glycans reported were unsialylated, 50% were monosialylated, and 13% were disialylated; additionally, 48% of the *N*-Glycans were fucosylated. A total of 10% of the glycans belonging to the minor group were reported as sulfated or phosphorylated.

Major and intermediate group compositions represented more than 80% of the total. Their average relative intensities ranged between 0.2 and 20%. All of the major glycoforms were reported previously (45-48): $Hex_5HexNAc_4dHexNeuAc$, $Hex_5HexNAc_4dHexNeuAc_2$, $Hex_6HexNAc_3NeuAc$, $Hex_5HexNAc_4NeuAc_2$,

$Hex_5HexNAc_4NeuAc$, $Hex_4HexNAc_3dHexNeuAc$, $Hex_4HexNAc_4dHexNeuAc$. All of the major compositions contained NeuAc, 2 contained 2 NeuAc residues, and 4 were fucosylated. Except for the hybrid glycans $Hex_6HexNAc_3NeuAc$ and $Hex_4HexNAc_3dHexNeuAc$, all were biantennary complex compositions. In the intermediate group, 7/11 glycans were sialylated and 4/11 were fucosylated. Except for $Hex_5HexNAc_2$, all were biantennary *N*-glycans. A total of 4/11 intermediate *N*-glycans corresponded to the compositional range $Hex_4HexNAc_5dHex_{0-1}NeuAc_{1-2}$. Three laboratories (14, 17 and 19(a)) proposed a degree of interpretation of the branching structure of the glycans. The structure of $Hex_4HexNAc_5NeuAc_1$ was described by laboratories 14 and 17 as an asymmetrical biantennary *N*-glycan containing the antenna: NeuAcα2-6GalNAcβ1-4GlcNAcβ1-2Man. Regarding the intermediate compounds, $Hex_4HexNAc_5dHexNeuAc$ was described by laboratories 14 and 17 as containing the same antennary structure. These assignments for the *N*-glycans correlate with those described in the literature (49-51). Laboratories 14 and 19(a) described $Hex_4HexNAc_3dHex_{0-1}NeuAc$ containing the same antenna. Triantennary $Hex_6HeNAc_5dHex_xNeuAc_y$, and $Hex_5HexNAc_5dHex_xNeuAc_y$ were observed within the class of minor compositions. Laboratories 14, 17 and 19(a) concluded that the majority of the PSA and PSA high isoform *N*-glycans were biantennary.

Three high mannose compositions were observed: $Hex_4HexNAc_2$, $Hex_5HexNAc_2$, $Hex_6HexNAc_2$, with PSA containing higher relative abundances than PSA high isoform. The most abundant high mannose composition was $Hex_5HexNAc_2$ (intermediate group) which was observed by 25% of the laboratories using top-down MS, 64% using bottom-up and 75% using PNGase F release. The relative intensities for the PNGase F released high mannose *N*-glycans was higher than those observed for analysis of bottom-up glycopeptides, possibly due to the fact that the high mannose isoforms were released from a contaminating protein in addition to PSA. The release of the *N*-glycans by PNGase enzymatic digestion does not distinguish the site of glycosylation and the glycan profile is the result of the *N*-glycans present on all glycoproteins in the samples. The purity of the sample and the number of glycosylation sites is

thus a major limitation of the approach of analyzing PNGase F released *N*-glycans.

Because the PSA is of human origin, NeuAc is the only sialic acid expected; however, laboratories 20, 21 and 15 reported presence of NeuGc. The compositions identified were not consistent and the relative intensities lower than 2%. The identifications were based on exact masses, not tandem MS data.

The presence of sulfated and/or phosphorylated *N*-glycans was reported by laboratory 24 using a HPAEC-PAD method. The *N*-glycans were first obtained by PNGase F release of PSA and PSA high isoform and then digested using non-specific neuraminidase and α(2-3)-specific neuraminidase enzymes. The HPAEC-PAD chromatograms indicated presence of a minor peak, consistent with a charged oligosaccharide with the potential to carry sulfate and/or phosphate substituents. The presence of sulfated and/or phosphorylated glycans were also detected for PSA and PSA high isoform by participants analyzing bottom-up (laboratories 6, 10, 9(a) and 16), PNGase F released glycans (laboratories 9(b) and 22) and top-down (laboratories 20, 21) data. Laboratories 20, 21 and 22 did not provide tandem MS evidence. The average intensity of this set of compounds was lower than 1%. The most abundant such composition $Hex_4HexNAc_5dHex(SO_3)$ was reported by laboratories 6, 9(a), 9(b), 10 and 16 for PSA and laboratories 7, 9(a) and 9(b) for the PSA high isoform. Except for $Hex_4HexNAc_5dHex_1Neu_1Ac(SO_3)$, the other sulfated/phosphorylated *N*-glycans were observed by fewer than three participants and, with the exception of laboratory 9, they were not observed in both PSA and PSA high isoform. We conclude that the consensus for identification of sulfated/phosphorylated glycans among the participants was not strong.

Quantitative Analysis of Glycan Isoforms on PSA and PSA high isoform

We conducted quantitative analysis of PSA and PSA high isoform data as follows. We derived a consensus data set using a preliminary AHC of the participant data. Next, a differential comparison of

the *N*-glycan profiles observed between PSA and PSA high isoform was obtained from the consensus data. Finally, a W-test was used to determine *N*-glycan compositions that differed significantly in abundance between PSA and PSA high isoform.

Agglomerative hierarchical clustering analysis

We used AHC of the complete dataset to compare results from participating laboratories for observation and quantification of *N*-glycans of PSA and PSA high isoform. The results (**Figure 1)** illustrate the multivariate analysis and clustering in two dimensions across all groups into four clusters (A, B, C, D), corresponding to: A (laboratory 8), B (laboratory 22), and D (laboratory 14, 4 and 13), representing a total of 5 experiments out of 22 of the participating laboratories, while cluster C contained the results from 17 laboratories. The cluster C results were the most uniform and were used to construct consensus data. In order to better understand the AHC results, we plotted the average of the intensity of each major and intermediate *N*- glycan composition for the four clusters, as shown in **Figure 2**.

Cluster A

Cluster A was comprised of a single set of results reported from laboratory 8. None of the major compositions were detected except for $Hex_4HexNAc_3dHexNeuAc$ at 63.5% for PSA and 4% for PSA high isoform. By comparison, this composition was observed with average intensity of 14.7% in cluster C. The compounds reported with the highest intensity were $Hex_4HexNAc_4dHexNeuAc$ (23.5%) and $Hex_6HexNAc_4NeuAc$ (68.5%) for PSA high isoform. The average intensities for the same compounds in the consensus cluster C were 2.8% and 5.2%, respectively. Altough the results disagreed with those of the consensus cluster, laboratory 8 reported that they performed comprehensive analysis including tandem MS experiments (both HCD and ETD) on glycopeptides resulting from a tryptic digestion using high resolution MS (R=60,000, mass accuracy 10 ppm). The tandem MS data were searched to identify

glycans using Byonic™ and SimGlycan™ software. The quantification was made using peptide NKSVILLGR.

Cluster B

Cluster B was comprised of a single set of results reported by laboratory 22 and differed significantly from the consensus cluster. The *N*-glycan composition $Hex_6HexNAc_3NeuAc$ was not reported for PSA and PSA high isoform while the average of the relative intensities for this composition in cluster C were respectively 14.7% for PSA and 1.1% for PSA high isoform. The $Hex_4HexNAc_3dHexNeuAc$ composition was not detected for PSA and a low intensity (0.1%) was observed for PSA high isoform. By comparison, in cluster C, the average relative intensity of this composition was 14.3% and 2.3% for PSA and PSA high isoform, respectively. The compositions $Hex_5HexNAc_4dHexNeuAc_2$ and $Hex_5HexNAc_4NeuAc_2$ differed in cluster B relative to cluster C by a factor ranging from 9 to 14 for PSA and 2 to 4 for PSA high isoform. The *N*-glycan composition $Hex_5HexNAc_4dHexNeuAc$ was lower for cluster B than cluster C by a factor of 9 for PSA and 13 for PSA high isoform. Among the intermediate compositions, only $Hex_4HexNAc_4NeuAc$ and $Hex_4HexNAc_3NeuAc$ for PSA were detected. The results also included some tri- and tetra-antennary compositions that were not observed by other participants. The samples were processed using PNGase F release, followed by LC-MS analysis. No tandem MS data were reported.

Cluster D

Cluster D contained results from laboratories 14, 4 and 13. The *N*-glycan profile contained distinct characteristics compared to the results reported in the consensus cluster C. In cluster D, the abundant *N*-glycan compositions $Hex_4HexNAc_4dHexNeuAc$ and $Hex_6HexNAc_3dHex$ for PSA high isoform and $Hex_6HexNAc_3dHex$ for PSA were not detected. The majority of the other compositions differed in intensity significantly between clusters D and C. For example, while the average intensities of the

compositions of the two most abundant major glycans ($Hex_5HexNAc_4dHexNeuAc$ and $Hex_5HexNAc_4dHexNeuAc_2$) were higher for the PSA high isoform than PSA for cluster C, they are nearly the same in cluster D. In cluster C, the $Hex_5HexNAc_4NeuAc_2$ composition was more intense in the PSA high isoform (4.3% compared to 2.3% for PSA) while the inverse was observed in cluster D (10.1% and 4.9%). The compositions $Hex_4HexNAc_4dHexNeuAc$, $Hex_4HexNAc_3dHexNeuAc$ and $Hex_6HexNAc_3NeuAc$ were more intense for PSA than PSA high isoform in cluster C compared to cluster D. Clusters C and D also differed in the observation of intermediate and minor compositions. About half the intermediate compositions observed in cluster C, including $Hex_4HexNAc_4dHex$, $Hex_4HexNAc_5NeuAc_2$, $Hex_3HexNAc_5dHexNeuAc$ and $Hex_4HexNAc_4NeuAc$ seen in PSA were not detected in cluster D. Many minor compounds were not observed for PSA or PSA high isoform in cluster D (7/43 and 9/43 compositions detected, respectively) compared to cluster C (39/43 and 37/43 compositions detected, respectively).

Cluster C

Results from 17 of the 22 participating laboratories were grouped to make up consensus cluster C. Two sub-clusters were observed: C1 (laboratories 2(a), 3, 6, 9(a), 9(b), 10, 16, 18(b), 19(b), 20 and 23) and C2 (laboratories 1, 2(b), 5, 7, 12 and 21). The differences between clusters C1 and C2 were small. The results are presented in **Supplemental Figure 5**. The average intensities of major and minor compounds between PSA and PSA high isoform are comparable for almost all the major and intermediates compounds in clusters C1 and C2 except for $Hex_4HexNAc_3dHex$ and $Hex_5HexNAc_4NeuAc_2$. We observed that if a compound was more intense for PSA compared with PSA high isoform for cluster C1, the tendency was the same for cluster C2. We observed that the differences of relative intensities between C1 and C2 did not exceed 10.8% for $Hex_5HexNAc_4dHexNeuAc_2$ in PSA high isoform.

Comparative Analysis of Applied Methodologies

Following the analysis of the primary clusters A through D we performed a more detailed evaluation of each method step including sample preparation, separation methods, choice of proteolytic enzyme and choice of MS method.

Separation methods used for the analysis of intact protein, glycans and glycopeptides

Peptides issued from the enzymatic digestion were separated by different types of chromatography: C_{18} reversed phase chromatography was used by laboratories (1, 2(a), 3, 4, 5, 6, 7, 8, 11, 13, 14, 15), C_8 reversed phase column by the laboratory 16, PGC column chromatography by laboratory 9(a), CE by 10. The glycans resulting from PNGase F digestion were separated by PGC chromtaography (laboratories: 2(b), 9(b), 22). The glycoprotein was separated by PLRP-S column for the laboratory 19(b), by RP-4H for 20, C_8 reversed phase chromatography for 21 and finally by CE for 22.

The diversity of chromatography methods used combined with the limited number of participating laboratories prevented establishment of a clear trend concerning the approaches; however, the results presented in clusters A and D were submitted by laboratories using C_{18} reversed phase chromatography.

Enzymatic digestion used to prepare glycopeptides

The choice of enzyme and digestion conditions (pH, ratio enzyme/glycoprotein) may impact the results of enzymatic digestion in term of nature and number of glycopeptides produced. In addition, there are two important considerations regarding PSA which will impact the results obtained by the different laboratories. The glycosylation site is in close proximity to two tryptic cleavage sites and PSA itself is a protease which can, if not handled properly, induce self-digestion. The susceptibility of the PSA peptide backbone to proteolytic cleavage may depend on the structure of the glycan present. Because such glycans represent a heterogeneous population, the effects on proteolytic cleavage pattern may impact glycopeptide quantification. Stavenhagen et al (52) proved clearly through the study of synthetized of

glycopeptides the impact of the nature glycopeptides on the free label quantification.

- Choice of enzyme

As with sample separation, we did not observe a discernible trend based on the type of proteolytic enzyme used. Again, this was due to the diversity of enzymatic digestion methods and the limited number of participants. We note that laboratory 8 (cluster A) reported using trypsin digestion and laboratories in cluster D reported using Arg-C, trypsin or both chymotrypsin and trypsin. The consensus cluster C contained results obtained using trypsin (8 laboratories), Lys-C (1 laboratory) and chymotrypsin (1 laboratory).

- Data Analysis

A trend was observed in the clustering data concerning the number of peptides in the bottom–up experiment that were used to quantify the different *N*-glycan compositions.

Among the participating laboratories in cluster D, laboratory 14 used two different peptides (NK and NKSVILLGR), laboratory 4 used 4 peptides (NKSVILLGR, GRAVCGGVLVHPQWVLTAAHCIRNK, AVCGGVLVHPQWVLTAAHCIRNK, and AVCGGVLVHPQWVLTAAHCIRNKSVILLGR) and laboratory 13 used 3 peptides (NKSVIL , RNKSVILL and NKSVILLGR). Cluster C contained results from 10 participating laboratories using a bottom-up approach; 7 of these used a single peptide for quantification. Laboratories 1, 5 and 7 used NKSVILLGR, laboratory 16 used HSQPWQVLVASRGRAVCGGVLVHPQWVLTAAHCIRNK, and 3, 9(a) and 10 used NK. We note that laboratory 9(a) performed quantification of PSA high isoform mainly on the peptide NK (80%) but also on the peptide NKSVILLGR (20%).

Results from laboratories 3, 9(a), 10 and 16 were grouped in cluster C1 and results from laboratories 1, 5

and 7 constituted cluster C2. The correlation between clustering and number of peptides used for quantification may result from the fact that the efficiency of ionization differs among peptides and/or differences in chromatographic separation efficiency. If the quantification of the *N*-glycans was performed using the same peptide(s) among different laboratories, error due to these effects would be minimized.

Choice of MS Method: Top-Down vs. Bottom –Up vs. PNGase F release

All of the participating laboratories using top-down analysis obtained comparable results that were grouped in cluster C. A total of 75% of the laboratories using PNGase F release and 71% of those using bottom-up analysis were also included in cluster C.

To determine if results were dependent upon the methods used, the average percentage for all laboratories observing a particular glycan composition was compared to the average percentage reported for the same compositions for a given method analysis (Top-down, bottom-up, PNGase F). For example, the compounds $Hex_5HexNAc_4NeuAc_1$ was detected by 82% of the total participating laboratories (18/22 participating laboratories), by 71% of the participants using bottom-up approach (12/17 laboratories) and by 100% of the participants using Top-down and PNGase approaches (4/4 laboratories). The results are presented in **Table 2**. We observed a clear trend in the major and intermediate groups of *N*-glycan compositions. The averaged abundances for *N*-glycan compositions for top-down and PNGase F released glycans were higher than the global average. Thus, the top-down and PNGase F approaches were more efficient in detecting the major and intermediate compositions than the bottom-up approach. A clear trend could not be established for the minor compositions.

The robustness among different methods, which we defined by the consistency in results, was compared by calculating the differences in average standard deviations. Permutation tests were used to

artificially extend the data set and to calculate the differences in standard deviation between different approaches. The method robustness was ranked using the p-values obtained between pairs of approaches. The results are shown in **Figure 3**. The red lines show the difference between the average standard deviations for the two methods in each plot. The p-values correspond to double the area of bars to the left of the red line in each plot. When all 22 participants were included the following p-values resulted: comparison between top-down and PNGase F release, p = 0.21; top-down and bottom-up, p = 0.15; PNGase F release and bottom-up, p =1.00. While the permutation test fails to provide statistical significance for this ranking of methods, the consistency of results according to this statistical measure may be ranked as follows: top-down ≥ PNGase F release> bottom-up. We note that top-down analysis is usually performed in dedicated mass spectrometry facilities with experience in this area and the methodology involves minimal sample manipulation, both of which may contribute to a lower occurrence of errors due to sample handling and preparation. We also note that laboratories which routinely perform PNGase F protocols tend to have considerable experience in the field of glycomics and may be more skilled in sample preparation and analyses for glycan characterization.

Using AHC, laboratories 8, 22, 13, 4 and 14 were excluded from the consensus data cluster C. These excluded participants used a bottom-up approach except for laboratory 22, which analyzed PNGase F released glycans. The same statistical treatment as described above was applied to the consensus data. We obtained improved adjusted p-values: top-down and PNGase F release p= 0.54, top-down vs. bottom-up p=0.31, PNGase F and bottom-up p=0.75. Again, while we are unable to establish statistical significance for this ranking mainly due to the limited numbers of data sets, our data in this subset show reliability rankings for these data sets as follows: top-down ≥ PNGase F release> bottom-up.

The following comments apply to the observed ranking of methods:

1. The PSA glycoproteins contains a single *N*-glycosylation site and a relatively low molecular

weight. Thus, it is likely that the ranking of methods would differ if a larger glycoprotein with more than one glycosylation site were studied. In such a case, use of top-down analysis would be more challenging. The analysis of released *N*-glycans would require prior purification of glycopeptides.

2. The consensus data set (cluster C) consisted of 17 laboratories using bottom-up, 4 using top-down, and 4 using PNGase F release. It will be of interest to confirm the conclusions of the present study using a larger number of participating laboratories using top-down and PNGase F release methods, respectively

N-Glycan Profiles of PSA and PSA high isoform

The ability to quantify glycoforms has the potential to increase the diagnostic sensitivity and specificity of the PSA cancer biomarker. The literature reports that the oligosaccharide profiles of PSA differ in healthy vs. prostate tumor metastatic cell line LNCaP (47, 53-54). The oligosaccharides of PSA from healthy patients include sialylated biantennary fucosylated complex glycoforms, some with antenna GalNAc. The presence of high mannose and hybrid *N*-glycans has also been reported. The GalNAc was observed in increased abundance in LNCaP glycans. Peracaula *el al.* reported that the LNCaP oligosaccharides were almost all neutral and contained high fucose content (53). Those from seminal fluid PSA were almost completely sialylated and contained a lower degree of fucosylation. However, Ohyama (45) *et al.* reported the presence of sialic acid in the PSA secreted by the LNCaP cells. These contradictory results may be due to differences in the cell growth conditions or deviation of cells from the parent cell line. Recently, PSA *N*-Glycans from tissues of healthy individuals were reported to be less sialylated than those of cancer patients (55). The authors conclude that *N*-glycans of PSA from seminal fluid of healthy patients is more sialylated than those from cancer patients (56). The α2-3 sialic acid linkage has also been reported to distinguish malignant from benign cancer (45-46) but the results are

not consistent among studies. A more recent study on PSA from prostate tissue from healthy and cancerous donors, highlighted that the sialylated glycoforms were elevated in cancer (55). Despite the clear evidence that glycan profiles differentiate healthy vs. cancer states, the analytical data show contradictory results regarding the most abundant glycans that are likely due to variability among PSA sources, the purity of the protein, and analytical methods used to profile the glycans. Developing analytical methods that allow reproducible characterization of relative abundances of *N*-glycans from different sources of PSA is thus a crucial step to improving the value of PSA as a biomarker.

Consensus N-glycan profiles of PSA and PSA high isoform

The consensus cluster C *N*-glycan profiles were etablished for PSA and PSA high isoform using data from participating laboratories (2(a and b), 3,5,6,7,9 (a and b), 10, 11, 12, 16, 18(b), 19(b), 20, 21 and 23). Among these, four participants used top-down, three PNGase F and ten used bottom-up methods.

The heat map in **Figure 4** shows the differences of relative intensities of *N*-glycan compositions between PSA and PSA high isoform. Hierachical clustering data were processed for the differences in relative intensities for *N*-glycan compositions in order to define which carbohydrates differ between PSA and PSA high isoform. **Figure 4** shows clearly that the intensities of more than 75% of the *N*-glycan compositions were not significantly different between PSA and PSA high isoform (cluster 7). Clusters 1,2,3,4,5,6 and 8 contained the *N*-glycan compositions for which relatively significant differences in relative intensities between PSA and PSA high isoform were observed. Clusters 1,3,4, and 6 contained *N*-glycan compositions with relative intensities higher in PSA high isoform than PSA; clusters 2,5, and 8 contain those lower for PSA high isoform than for PSA. A Wilcoxon rank-sum statistical test on the consensus data determined that 8 of the 18 major and intermediate compositions differed significantly in relative abundances (p value <0.0008). Four *N*-glycan compositions ($Hex_4HexNAc_4dHexNeuAc$, $Hex_4HexNAc_3dHexNeuAc$, $Hex_6HexNAc_3NeuAc$, $Hex_4HexNAc_3NeuAc$) were higher in relative abundances

in PSA than in PSA high isoform. The difference between PSA and PSA high isoform of the average relative intensities for those compounds ranged between 2.6% and 13.8%. Four compositions ($Hex_5HexNAc_4dHexNeuAc_2$, $Hex_5HexNAc_4dHexNeuAc$, $Hex_4HexNAc_5dHexNeuAc_2$, $Hex_5HexNAc_4dHex$) were reported more intense in PSA high isoform than PSA with a difference of average relative intensity ranging from 2.4% to 22.2%, **Figure 5**.

The consensus of the average relative intensities of the major and intermediate *N*-glycan compositions allowed us to distinguish the differences with regard to sialylation and fucosylation of PSA and PSA high isoform, **Table 3**. The PSA was less disialylated and fucosylated than PSA high isoform. The two samples had the same percentage of asialo compositions, approximately 5%. Finally, the most abundant PSA and PSA high compositions corresponded to biantennary *N*-glycans.

Conclusion

The three analytical strategies employed by participant laboratories for comparative analysis of PSA samples were top-down analysis of the glycoprotein, bottom-up analysis of glycopeptides, and analysis of PNGase F released *N*-glycans. Consensus cluster C representing 17 of the 22 participating laboratories produced consistent results for comparison of *N*-glycan compositional profiles between PSA and PSA high. Of the 61 *N*-glycan compositions in the consensus data, 8 differed significantly in abundance between the two PSA samples. The data demonstrated that disialylated and fucosylated compositions are higher in abundances in the PSA high isoform than the PSA sample.

These results highlight the value of top-down MS for characterization of glycoprotein containing one glycosylation site. The advantage of top-down MS is that there is no need for proteolytic digestion and subsequent workup. The major disadvantage of top-down is that the glycoprotein is observed as a distribution of precursor ion *m/z* values corresponding to the heterogeneous glycoforms present. This

heterogeneity divides the ion signal. Nonetheless data from all participants using top-down MS (4/22 laboratories) were represented in the consensus cluster C. We conclude that there is potential for top-down for analysis of glycoproteins containing one glycosylation site; however, future studies will be required to determine whether top down perform effectively in analyses with multiple sites of glycosylation.

Among laboratories using bottom-up glycoproteomics, 71% are represented in the consensus data set (cluster C). The strongest factor that correlated with the consistency of bottom-up results was the number of peptide backbone sequences used to quantify the glycan compositions. A total of 70% of the participants using bottom-up in cluster C used a single sequence for quantification. Among the participant bottom-up data not represented in the consensus cluster, all used more than one peptide sequence for glycan composition quantification. We conclude that a combination of factors makes combining glycosylation quantitative data from more than one peptide sequence challenging. Among these are false positive identifications and glycopeptide ionization efficiencies. It is clear that great care must be taken with regard to the selection of proteases, digestion conditions, and peptides used for comparative glycosylation studies using glycopeptides.

A total of 75% of the laboratories analyzing PNGase F released glycans were included in the consensus data set. The ability to accurately characterize the abundances of *N*-glycan compositions was judged to be similar to that for top-down and better than bottom-up analysis. It must be noted, however, that analysis of released *N*-glycans for an intact glycoprotein involves the assumption that the observed compositions derive from the target glycoprotein. Prior to the present study, we evaluated several glycoproteins from commercial sources; many of these contained high levels of proteins other than the target. For the PSA and PSA-high isoform samples used in this study, other proteins were present at approximately 10%. Other sources of PSA were considerably less pure. It therefore seems unwise to

assume glycoprotein purity when designing an analytical strategy. We conclude that PNGase F release is best used on a glycoprotein of verified purity.

The application of top-down and glycan release methods becomes more challenging as the size of the glycoprotein, number of glycosylation sites, and heterogeneity grows. For large, complex glycoproteins, top-down analysis is presently limited to molecular weight profiling with glycosidase digestion to determine extent of total glycosylation. Top-down tandem MS will be considerably more challenging for such glycoproteins since they typically will be more heterogeneous than PSA. Analysis of released glycans is appropriate for peptide chains that carry only one glycosylation site. Such peptides may be purified in favorable cases. Thus, use of proteolytic enzymes is likely to be necessary for glycoprotein analysis, despite the fact that bottom-up methods suffered from reproducibility problems in the present study.

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List of Figures.

Figure 1. Agglomerative hierarchal clustering of N-glycans profiles for PSA and PSA high isoform from the participating labs. The dendrogram illustrates by clustering (X axis) and height (Y axis) the degree of similarity of profile of different N-glycans of PSA and PSA high Isoform detected by different labs. The main clusters reported are: (A), (B), (C), (D), and subclustering could be distinguished (C_1), (C_2).

Figure 2. Average relative intensity of each compound per cluster for the major, intermediate and minor *N*-glycans. Clusters A (Lab 8) and B (lab22) showed a complete difference with the other participating laboratories in term of repartition for PSA and PSA high isoform. Lab 8 reported $Hex_4HexNac_3dHex_1Neu_1$ as the major compound (> 60%) for the PSA sample, while clusters C and D reported the same compounds with an intensity lower than 15%. Lab 8 did not report any of the major *N*-glycans that the other labs observed for PSA High Isoform and PSA except the $Neu_1dHex_1Hex_4HexNac_3$. The Cluster B for PSA and PSA high Isoform presented higher intensity for *N*-glycans $Hex_5HexNac_3Neu_2$ and $Hex_5HexNac_4dHex_1Neu_2$ than the other clusters C and D. Only cluster C was able to detect the intermediate *N*-glycans. In cluster A, the average relative intensity of the compound $dHex_1Hex_5HexNac_4$ is 23.5% while it is lower than 6% for all the other clusters. (b) Minor *N*-glycans. The main difference is observed for the compound $Hex_6HexNac_4NeuAc_1$ of the PSA high isoform that is detected by cluster A with a relative intensity of 68% compared to less than 1% for the other clusters.

Figure 3. Comparison of robustness for bottom-up, top-down and PNGAse F release methods used by the participating laboratories . (A) Data from 22 participating laboratories were included in the permutation tests. (B) Data from consensus cluster C were used in the permutation tests. The red lines show the difference between the average standard deviations for the two methods in each plot. The p-values correspond to double the area of bars to the left of the red line in each plot.

Figure 4. Heat map of the consensus data. Agglomerative hierarchical clustering results were processed based on the differences of relative intensity values for glycan compositions between PSA and PSA high isoform. Orange color indicates a null difference of relative intensity between PSA and PSA high isoform, yellow indicates a positive difference, red indicates a negative difference. The *N*-glycan compositions of cluster 7 (80% of the total compounds) are similar between PSA and PSA high isoform. Compositions are more intense in PSA high isoform than PSA for the cluster 1 (**$Hex_5HexNAc_4dHexNeuAc_2$** , **$Hex_5HexNAc_4dHexNeuAc$**), cluster 3 (**$Hex_4HexNAc_5dHexNeuAc_2$**) , cluster 4 ($NeuAc_1Hex_5HexNAc_4NeuAc$), cluster 6 (**$Hex_5HexNAc_4dHex$** , $NeuAc_2Hex_5HexNAc_4NeuAc_2$). Compositions are less intense in PSA high isoform than PSA for cluster 2 (**$Hex_4HexNAc_4dHexNeuAc$**, **$Hex_4HexNAc_3dHexNeuAc$, $Hex_6HexNAc_3NeuAc$**), cluster 5 ($Hex_4HexNAc_4NeuAc$, $Hex_5HexNAc_2$), cluster 8 **(**$Hex_3Hex_5dHexNeuAc$, **Hex_4Hex_3NeuAc**). In order to confirm the significance of the differences in *N*-glycan composition abundances between PSA and PSA high isoform, a W-test was used and revealed 8 significant compositions (indicated in bold).

Figure 5. Differential profile of *N*-glycan profilederived from the consensus data (Cluster C). A W-test was employed in order to determine 8 *N*-glycans (p value <0.0008)significantly different between PSA and PSA high isoform. The significant *N*-glycans are marked by **.

Table 1. Summary of analytical approaches and instrumentation used by the participating laboratories

	Enzyme	Chrom.	Lab.	Instrument	MS/MS	Res/Mass Acc	Workup (Rep)
(A) Bottom-up analysis of glycopeptides	Tryp	C_{18}	1	**AB SCIEX, QstarElite, Q-TOF	CID	15,000; 20ppm	1 (2)
			2(a)	**Bruker, AmazonETD, Ion Trap	CID	5,000/160 ppm	2
			3	Thermo, LTQ-FT Ultra	CID	25,000	1 (2)
			4	Thermo, LTQ-FT Ultra	SID	200,000/0.2	1 (10)
			5	Thermo,LTQ Orbitrap XL	CID/HCD	60,000	1 (4)
			6	Thermo,LTQ Orbitrap Velos	CID/HCD	15,000/<5ppm	3
			7	Thermo,LTQ Orbitrap Velos	CID/HCD	60,000/<3ppm	1 (5)
			8	Thermo,LTQ Orbitrap Elite	HCD/ETD	60,000/<10 ppm	2
		PGC	9(a)	Bruker,HCT 3D, Ion Trap	CID	5,000/0.1-0.3 Da	1 (1)
		CE	10	Bruker, Maxis Quad, Q-TOF	CID	40,000/1 mDa	1 (1)
	Chym	C_{18}	11	Thermo, LTQ-FT	CID	100,000/10ppm	1 (2)
			12	AB SCIEX, Qstar Elite, Q-TOF	CID	12,000/15ppm	3
	Tryp-Chym		13	Thermo,LTQ Orbitrap XL	CID	30,000/5ppm	2
	ArgC		14	*Waters, QTOF Premier, Q-TOF	CID	10,000/30ppm	1 (1)
			19(a)[1]	Bruker, AmazonETD, Ion Trap	CID	7,000/0.5Da	2(3)
	ArgC-Tryp		15	Thermo, LTQ	-	-	1 (2)
	LysC	C_8	16	Bruker, Maxis 4G, Q-TOF	CID	45,000/<0.02Da	1 (1)
		C_{18}	18(a)[1]	Thermo, Orbitrap Elite	ETD/HCD	240,000/<5ppm	-
	Lysyl Endo	-	17	**ABI Voyager DEPro MALDI-TOF	-	480/0.5 Da	-
(B) Top-down	-	PLRP-S	18(b)	Thermo, Orbitrap Elite	ETD/HCD	240,000/<10ppm	2
		RP-4H	19(b)	Bruker, Maxis 4G, Q-TOF	-	40,000/10 ppm	1 (2)
		C_8	20	Bruker, Maxis, Q-TOF	-	40,000/<0.2Da	1 (1)
		CE	21	Bruker, micrOTOF-Q	-	15,000/1Da	1 (1)
(C) Analysis of PNGAse F released glycans	PNGase F, Red	PGC	9(b)	Agilent, MSD XCT 3D, Ion Trap	CID	5,000/<0.3 Da	1
	PNGase F, Red	PGC	2(b)	Bruker, AmazonETD, Ion Trap	CID	>2,000/ 50 ppm	2
	PNGase F	PGC	22	Agilent, TOF 4224	-	10,000/<2ppm	2 (2)
	PNGase F Permet	-	23	Perspective Biosystem Voyager DE-RF, MALDI TOF	-	10,000/<0.2 Da	1 (3)
	PNGase F and other enzymes ***	HPAEC	24	Pulsed Amperometric Detection	-	Identification via standards	1 (2)

* Glycopeptides were enriched using a sialic acid capture-and-release protocol [27] where sialic acid is a prerequisite for the enrichment but is also removed during the procedure.

** ZIC-HILIC or HILIC cleanup

*** C30 Clean up

**** Other enzymes used were α2-3 sialidase, non-specific neuraminidase, Neu5Ac.

[1] Laboratory 18 and 19 carried out a bottom-up approach to confirm qualitative results obtained with their top-down approach. No quantitative data were submitted

Table 2. Percentage of participating laboratories detecting *N*-glycan. The percentage of *N*-glycan detected has been calculated by methods used (bottom-up, top-down, PNGase). For example, the compounds $Hex_5HexNAc_4NeuAc_1$ was detected by 82% of the total participating labs (18/22 participating labs), by 71% of the participants using bottom-up approach (12/17 labs) and by 100% of the participants labs using Top-down and PNGase approaches (4/4 labs). The green arrow indicates the percentage of *N*-glycan detected per methodology is higher than the percentage of *N*-glycan detected for all the participants, the yellow arrow indicate that the both percentages are the same, the red arrow indicates the percentage of *N*-glycan detected per methodology is lower than the percentage of N-Glycans detected by all the participating laboratories. The top-down and the PNGase methodologies presented better efficiency of detection than bottom-up for the major and intermediate N-glycan. The minor compounds did not present any such trend.

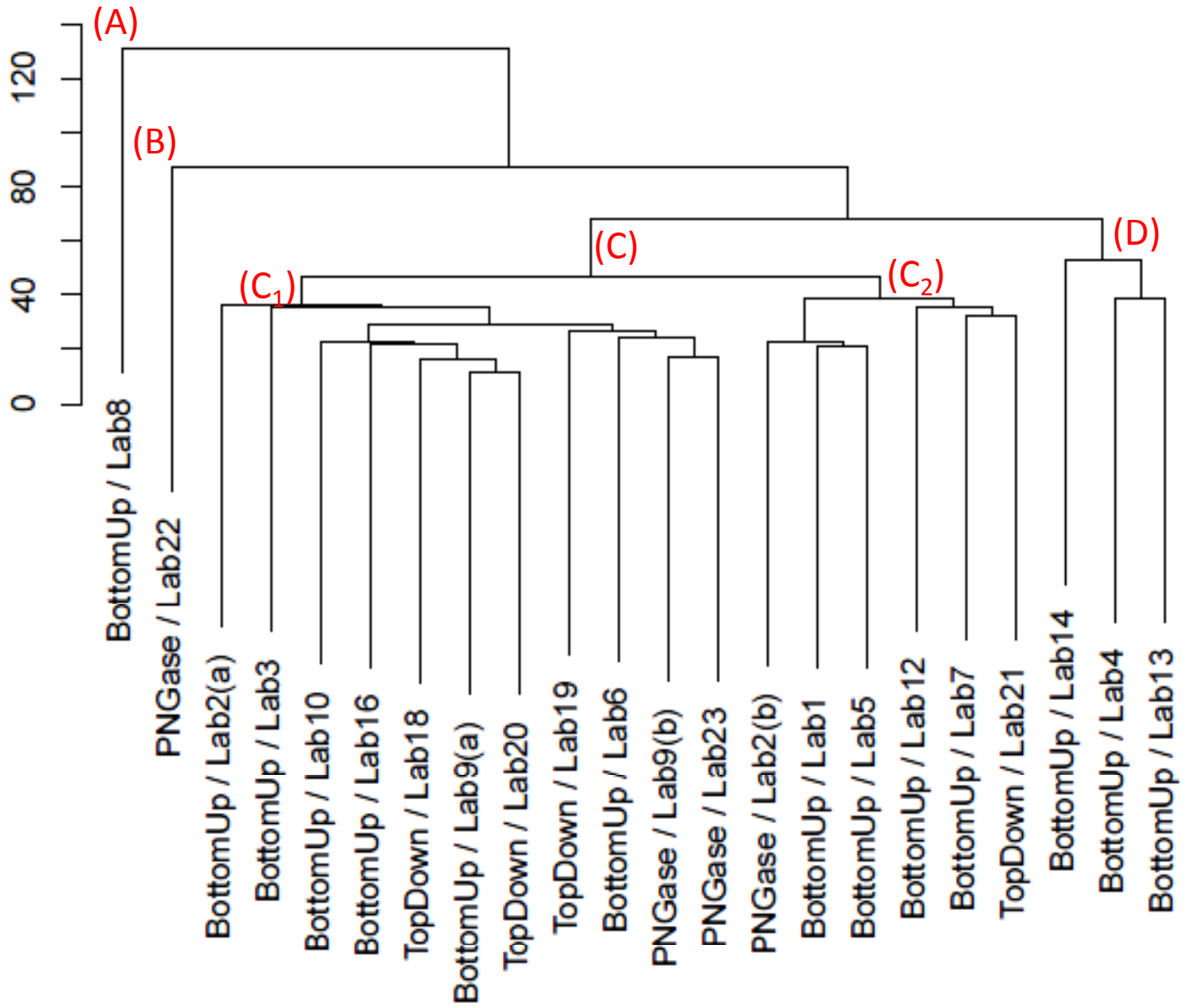
Class	Composition	% Participating laboratories	%Bottom up	%Top-down	% PNGAse
Major Glycan	$Hex_5HexNAc_4dHex_1NeuAc_1$	95	93	100	100
	$Hex5HexNAc_4dHex1NeuAc_2$	93	89	100	100
	$Hex_5HexNAc_4NeuAc_2$	84	75	100	100
	$Hex_5HexNAc_4NeuAc_1$	82	71	100	100
	$Hex_4HexNAc_3dHex_1NeuAc_1$	80	75	88	88
	$Hex_4HexNAc_4dHex_1NeuAc_1$	80	71	88	100
	$Hex_6HexNAc_3NeuAc_1$	68	64	75	75
Intermediate N-Glycan	$Hex_4HexNAc_5dHex_1NeuAc_2$	61	61	63	63
	$Hex_5HexNAc_4dHex_1$	61	64	75	38
	$Hex_4HexNAc_5 NeuAc_1dHex_1$	57	46	100	50
	$Hex_4HexNAc_3NeuAc_1$	57	43	63	100
	$Hex_4HexNAc_5NeuAc_1$	55	43	63	88
	$Hex_4HexNAc_4NeuAc_1$	52	43	63	75
	$Hex_4HexNAc_3dHex_1$	52	50	50	63
	$Hex_3HexNAc_5dHex_1NeuAc_1$	41	39	50	38
	$Hex_5HexNAc_2$	34	25	25	75
	$Hex_4HexNAc_4dHex_1$	34	36	25	38
	$Hex_4HexNAc_5NeuAc_2$	34	25	50	50
Minor N-Glycan	$Hex_5HexNAc_3NeuAc_1$	27	14	63	38
	$Hex_3HexNAc_5NeuAc_1$	25	25	25	25
	$HexNAc_5dHex_1NeuAc_1Hex_4SO_3$	23	21	13	38
	$Hex_4HexNAc_5dHex_1$	23	25	25	13
	$Hex_6HexNAc_2$	23	4	25	88
	$Hex_6HexNAc_3$	23	21	50	0
	$Hex_5HexNAc_4$	20	25	0	25
	$Hex_5HexNAc_5NeuAc_2$	20	11	63	13
	$Hex_4HexNAc_5$	20	11	50	25
	$Hex_5HexNAc_5dHex_1NeuAc_1$	20	18	50	0
	$Hex_3HexNAc_4dHex_1NeuAc_1$	20	14	38	25
	$Hex_6HexNAc_3dHex_1NeuAc_1$	20	29	0	13
	$NeuAc_1Hex_6HexNAc_4$	18	14	50	0
	$Hex_4HexNAc_2$	18	14	0	50
	$Hex_4HexNAc_5dHex_1SO_3$	18	21	0	25
	$Hex_5HexNAc_4dHex_2NeuAc_1$	18	21	0	25
	$Hex_3HexNAc_6dHex_1NeuAc_1$	16	11	38	13
	$Hex_5HexNAc_5$	14	14	0	25
	$Hex_6HexNAc_5dHex_1NeuAc_2$	14	11	13	25
	$Hex_6HexNAc_4NeuAc_2$	14	4	50	13
	$Hex_6HexNAc_5dHex_1NeuAc_3$	14	7	25	25
	$Hex_5HexNAc_5NeuAc_1$	14	14	25	0
	$Hex_4HexNAc_5NeuAc_1SO_3$	14	14	0	25
	$Hex_3HexNAc_6NeuAc_1$	14	14	13	13
	$Hex_3HexNAc_3dHex_1$	11	18	0	0
	$Hex_3HexNAc_4dHex_1$	11	18	0	0
	$Hex_5HexNAc_5 dHex_1$	11	4	50	0
	$Hex_6HexNAc_3 dHex_1$	11	4	38	13
	$Hex_3HexNAc_3dHex_1NeuAc_1$	9	14	0	0
	$Hex_3HexNAc_4dHex_1SO_3$	9	7	25	0
	$Hex_3HexNAc_5SO_3$	9	7	0	25
	$Hex_5HexNAc_2NeuAc_1dHex_2$	9	11	0	13
	$Hex_3HexNAc_5(SO3)_2$	7	4	0	25
	$Hex_4HexNAc_4NeuAc_1SO_3$	7	4	0	25
	$Hex_6HexNAc_4dHex_1$	7	0	38	0
	$Hex_6HexNAc_5$	7	0	13	25
	$HexNAc_1$	7	0	38	0
	$Hex_7HexNAc_3NeuAc_1$	7	11	0	0
	$Hex_4HexNAc_5dHex_2NeuAc_1$	7	11	0	0
	$Hex_4HexNAc_4NeuAc_2$	5	0	0	25
	$Hex_5HexNAc_4dHex_1NeuAc_1NeuGc_1$	5	0	25	0
	$Hex_5HexNAc_4SO_3$	5	4	0	13
	$Hex_4HexNAc_4NeuAc_2$	5	0	0	25

Table 3 Reported percentage repartition of un, mono,and disyalylated and fucosylated and unfucosylated *N*-glycans for the major and intermediate compounds. The significant *N*-glycans were revealed by the W- test as composition significantly different between PSA and PSA high isoform. PSA high isoform was more disialylaled than the PSA, when PSA is more monosialylated. The percentage of unsialylated compounds is relative comparable for PSA and PSA high isoform.PSA high isoform is more fucosylated than the PSA.

	Σ(Average Intensity N-glycans)		Σ (Average Intensity Significant N-glycans	
N-glycans	PSA	PSA High Isoform	PSA	PSA high isoform
Unsalylated	4.4	4.5	0.3	2.8
Mono Salylated	71.2	43.5	57.4	32.5
Disalylsated	8.3	37.9	5.7	33.0

	Σ(Average Intensity N-glycans)		Σ (Average Intensity Significant N-glycans	
N-glycans	PSA	PSA High Isoform	PSA	PSA High Isoform
Fucosylated	53.4	72.9	45.9	67.1
Unfucosylated	30.5	13.0	17.7	1.3

1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PNGase / Lab9(b)
PNGase / Lab23
PNGase / Lab2(b)
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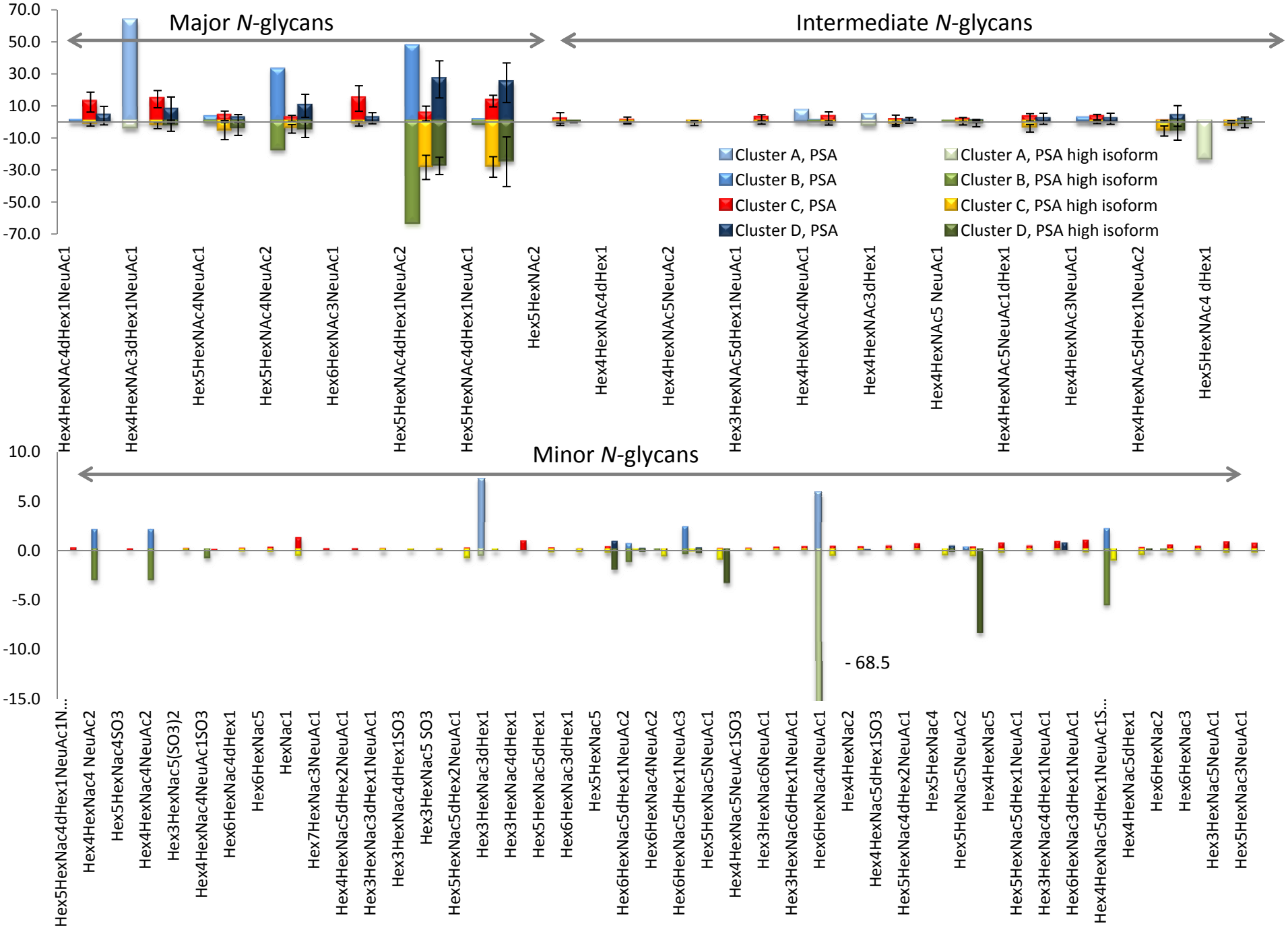
2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Intermediate N-glycans
Minor N-glycans
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-70.0
10.0
5.0
0.0
-5.0
-10.0
-15.0
- 68.5
Cluster A, PSA
Cluster B, PSA
Cluster C, PSA
Cluster D, PSA
Cluster A, PSA high isoform
Cluster B, PSA high isoform
Cluster C, PSA high isoform
Cluster D, PSA high isoform
Hex4HexNAc4dHex1NeuAc1
Hex4HexNAc3dHex1NeuAc1
Hex5HexNAc4NeuAc1
Hex5HexNAc4NeuAc2
Hex6HexNAc3NeuAc1
Hex5HexNAc4dHex1NeuAc2
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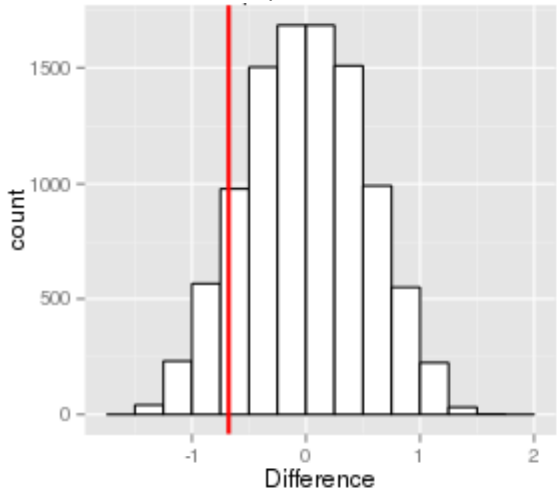
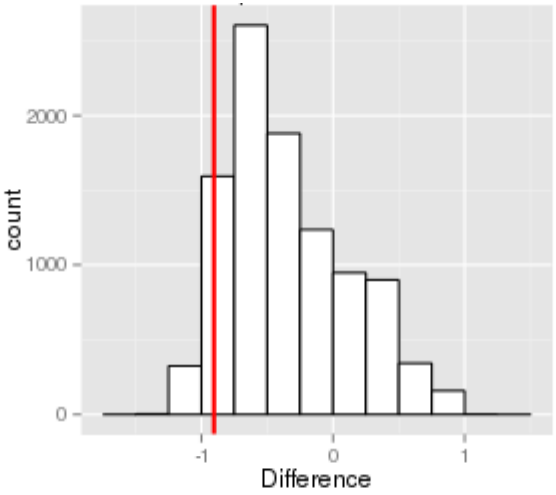
Hex3HexNac4dHex1SO3
Hex3HexNac5 SO3
Hex5HexNac5dHex2NeuAc1
Hex3HexNac3dHex1
Hex3HexNac4dHex1
Hex5HexNac5dHex1
Hex6HexNac3dHex1
Hex5HexNac5
Hex6HexNac5dHex1NeuAc2
Hex6HexNac4NeuAc2
Hex6HexNac5dHex1NeuAc3
Hex5HexNac5NeuAc1
Hex4HexNac5NeuAc1SO3
Hex3HexNac6NeuAc1
Hex3HexNac6dHex1NeuAc1
Hex6HexNac4NeuAc1
Hex4HexNac2
Hex4HexNac5dHex1SO3
Hex5HexNac4dHex2NeuAc1
Hex5HexNac4
Hex5HexNac5NeuAc2
Hex4HexNac5
Hex5HexNac5dHex1NeuAc1
Hex3HexNac4dHex1NeuAc1
Hex6HexNac3dHex1NeuAc1
Hex4HexNac5dHex1NeuAc1S...
Hex4HexNac5dHex1
Hex6HexNac2
Hex6HexNac3
Hex3HexNac5NeuAc1
Hex5HexNac3NeuAc1

3

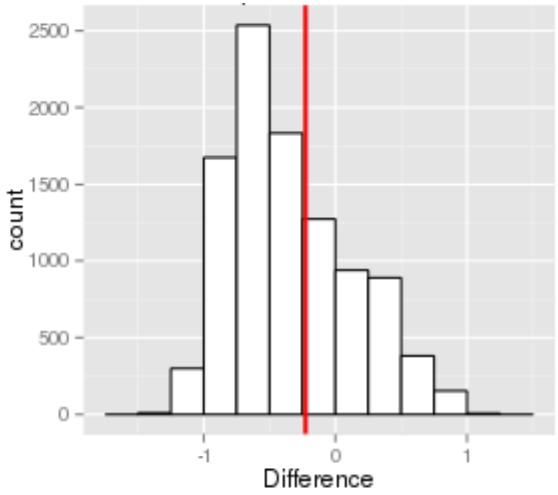
(A) (a) Avg SD : Top-down/PNGAse
p value : 0.21



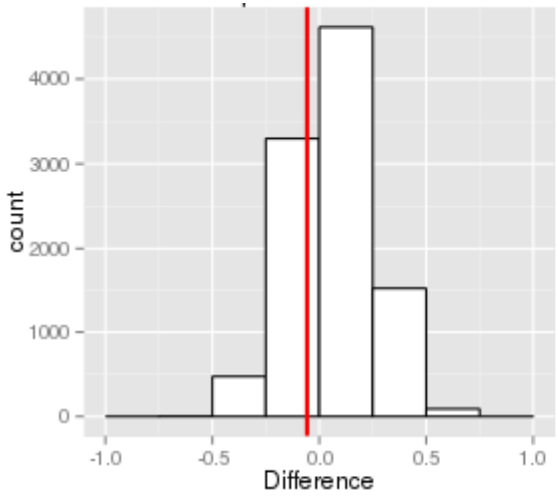
(b) Avg SD : Top-down/ Bottom-up
p value : 0.15



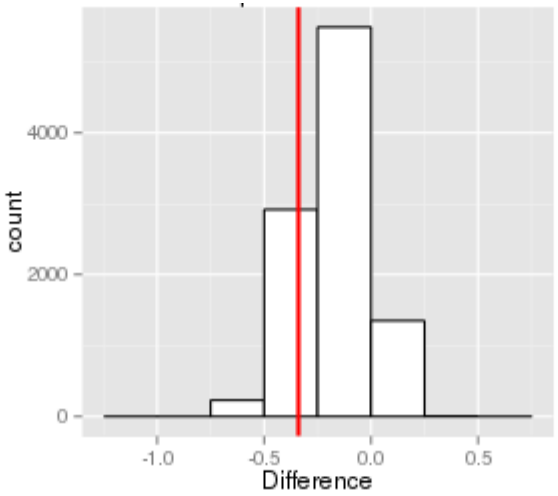
(c) Avg SD : PNGAse/Bottom-up
p value : 1



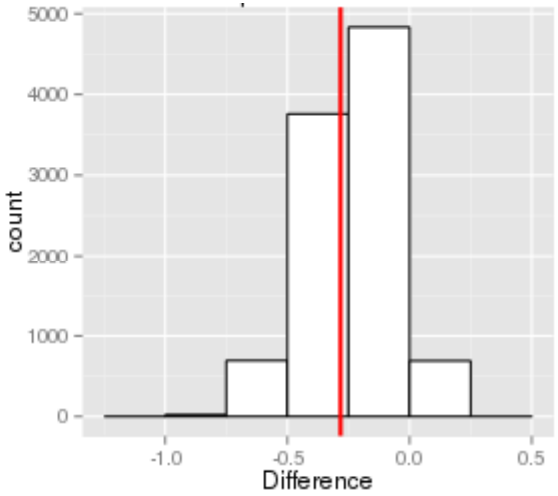
(B) (a) Avg SD : Top-down/PNGAse
p value : 0.54



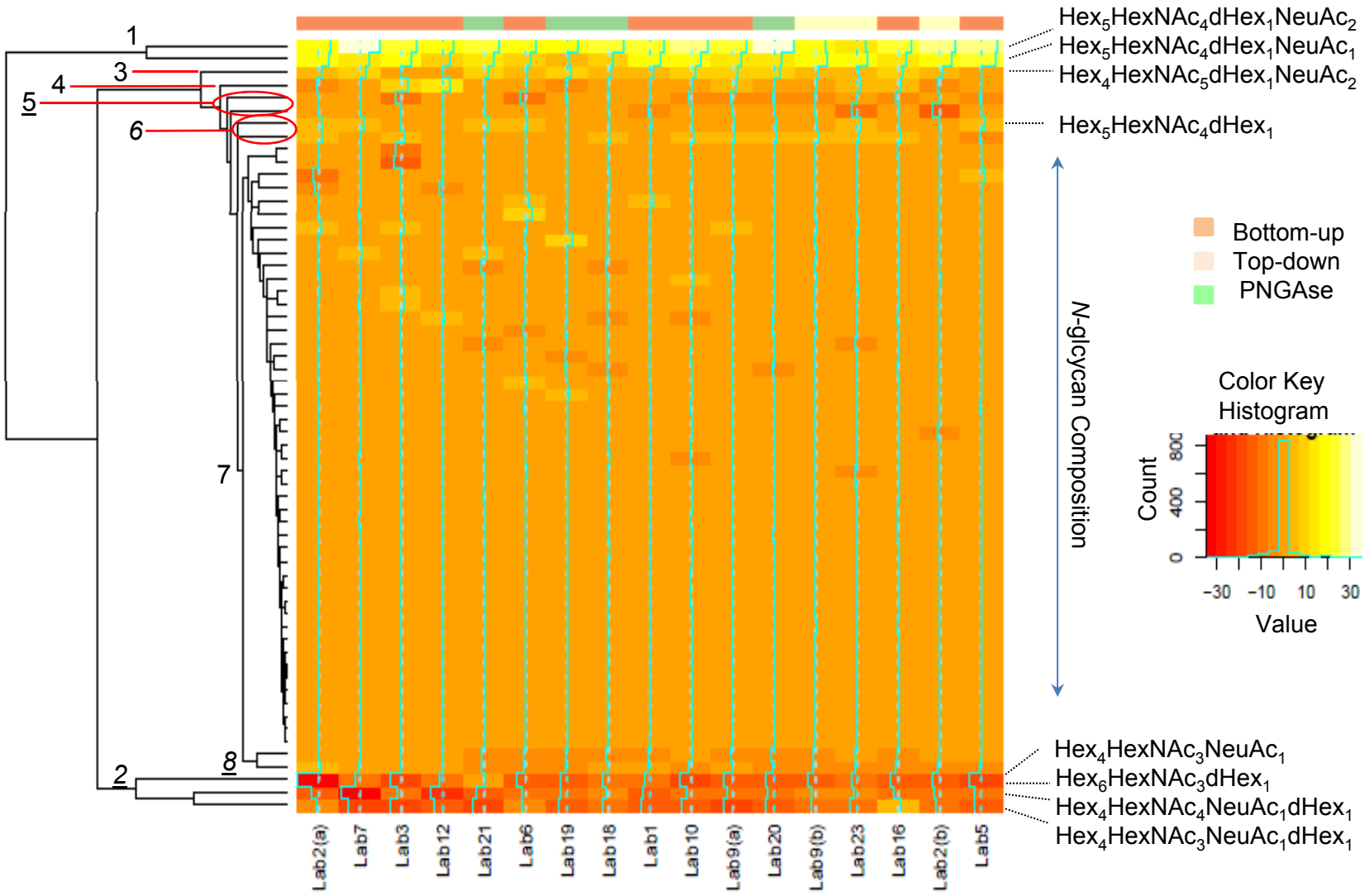
(b) Avg SD : Top-down/ Bottom-up
p value : .31



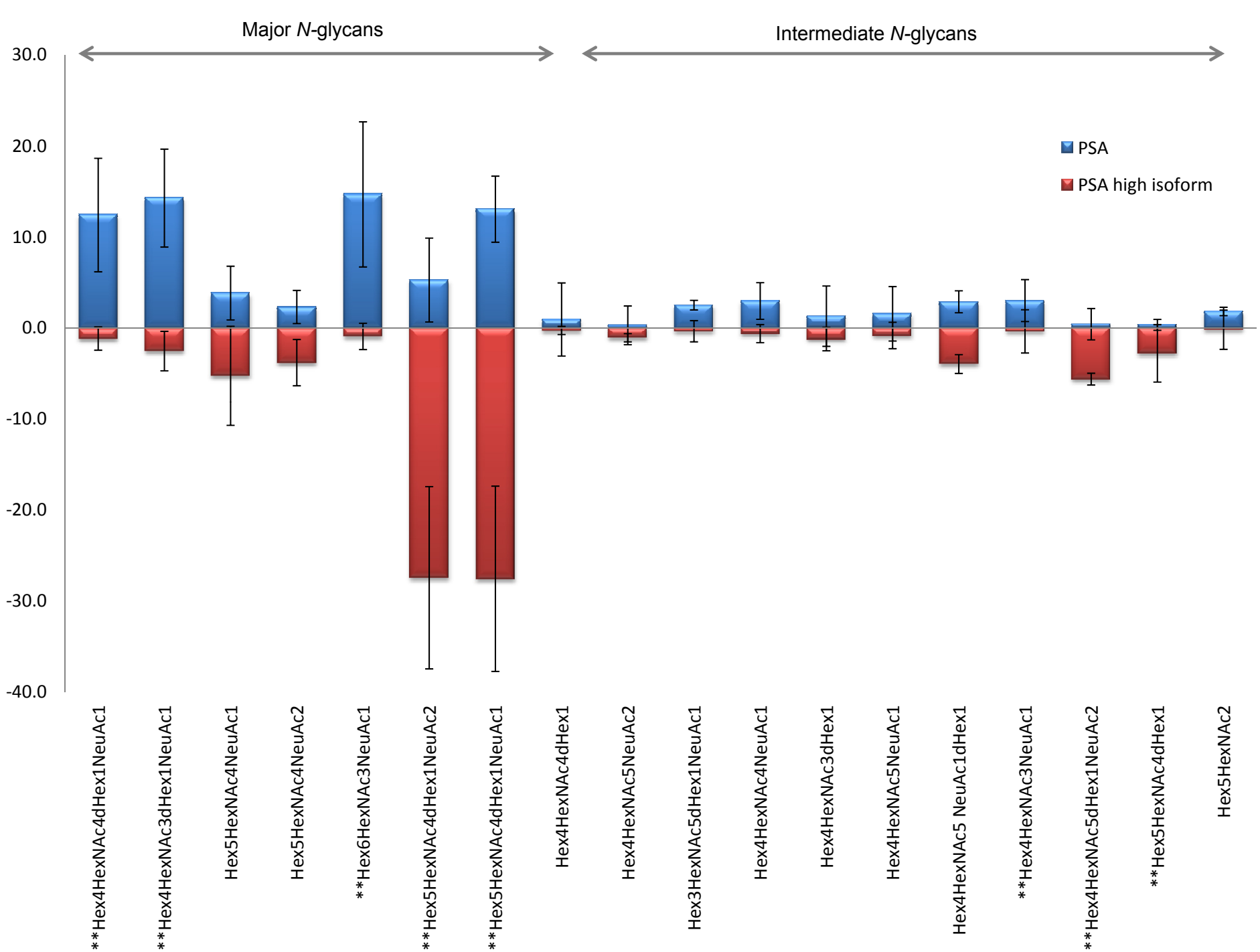
(c) Avg SD : PNGAse/Bottom-up
p value : 0.75



4


1
3
4
5
6
7
8
2
Hex5HexNAc4dHex1NeuAc2
Hex5HexNAc4dHex1NeuAc1
Hex4HexNAc5dHex1NeuAc2
Hex5HexNAc4dHex1
N-glcycan Composition
Bottom-up
Top-down
PNGAse
Color Key
Histogram
Count
0
400
800
−30
−10
10
30
Value
Hex4HexNAc3NeuAc1
Hex6HexNAc3dHex1
Hex4HexNAc4NeuAc1dHex1
Hex4HexNAc3NeuAc1dHex1
Lab2(a)
Lab7
Lab3
Lab12
Lab21
Lab6
Lab19
Lab18
Lab1
Lab10
Lab9(a)
Lab20
Lab9(b)
Lab23
Lab16
Lab2(b)
Lab5

5


Major N-glycans
Intermediate N-glycans
30.0
20.0
10.0
0.0
-10.0
-20.0
-30.0
-40.0
PSA
PSA high isoform
**Hex4HexNAc4dHex1NeuAc1
**Hex4HexNAc3dHex1NeuAc1
Hex5HexNAc4NeuAc1
Hex5HexNAc4NeuAc2
**Hex6HexNAc3NeuAc1
**Hex5HexNAc4dHex1NeuAc2
**Hex5HexNAc4dHex1NeuAc1
Hex4HexNAc4dHex1
Hex4HexNAc5NeuAc2
Hex3HexNAc5dHex1NeuAc1
Hex4HexNAc4NeuAc1
Hex4HexNAc3dHex1
Hex4HexNAc5NeuAc1
Hex4HexNAc5 NeuAc1dHex1
**Hex4HexNAc3NeuAc1
**Hex4HexNAc5dHex1NeuAc2
**Hex5HexNAc4dHex1
Hex5HexNAc2