



Differential Analysis of Glycan Populations of Monoclonal Antibodies Using Agilent mAb-Glyco Chip and Mass Profiler Professional

Application Note

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Abstract

This Application Note analyzes the glycan population of monoclonal antibodies (mAbs) produced under different growth conditions. The Agilent mAb-Glyco chip, coupled to an Agilent accurate mass Q-TOF LC/MS, was used. The mAb-Glyco chip permitted in-line deglycosylation and enrichment of liberated glycan moieties by porous graphitized carbon. Glycans were analyzed on an Agilent 6530 Q-TOF LC/MS, and data were processed initially in Agilent MassHunter Software with Bioconfirm and the Personal Compound Database and Library (PCDL) manager. The resulting data were imported into Agilent MassHunter Profinder for targeted recursive feature finding, and finally Mass Profiler Professional (MPP) for differential analysis. Use of MPP showed patterns in the data, and ultimately led to sample grouping clearly distinguishing mAbs produced under the different growth conditions.



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Introduction

Polysaccharides or glycans play important roles in a variety of biological processes. Glycoproteins are proteins covalently modified with glycan moieties. With the recent focus on antibody-based therapeutics, the study of the glycan modifications of these therapeutic molecules is an important part of their characterization. These modifications play a role in antibody stability, structure, biological activity, circulatory half-life, and appropriate effector function. There is a wealth of literature establishing that glycan modification patterns are sensitive to changes in pH, media, and growth conditions. These parameters can affect the extent of glycosylation, the degree of glycan branching, and the completeness of sialylation, which can ultimately affect antibody function.

The Agilent mAb-Glyco chip is a very convenient tool for studying the sugar populations of mAbs. The chip is simple to operate, allowing for in-line deglycosylation and glycan capture, which reduces sample handling and processing time, thus, improving reproducibility. Obtaining information about glycan populations is relatively straightforward. However, the downstream data analysis can be daunting as there is no straightforward way to compare the glycan populations of two antibodies, or automation to distill the data into a simple document.

In this study, we were interested in characterizing the glycan populations of antibodies that displayed differential binding by ELISA. These antibodies were produced from the identical cell type, but grown under different conditions. One set of antibodies was harvested from hybridoma cells grown in culture, while the other set was implanted in mice and subsequently harvested from ascites fluid. We were looking for an approach to simplify the data generated with the Find by Feature algorithm in Agilent MassHunter Qualitative Analysis and examine the data in a way that would allow us to detect differences among the glycan populations of different antibodies. Ultimately, we were hoping to uncover glycan differences that may explain the differential binding observed by ELISA. We found that importing the data into Agilent Mass Profiler Professional allowed us to consider all datasets and look for trends in an unbiased way. Using this approach, we were able to observe that the main source of variance in the glycan population was due to the production source of the antibody. We were also able to identify two specific glycans that were unique to our hybridoma antibodies.

Experimental

Materials

Immunoglobulin G (IgG) antibodies were obtained from either Aragen Bioscience Inc. (Morgan Hill, CA) or prepared internally by Gilead Sciences, Inc. and not commercially available.

Instrumentation

An Agilent 1260 HPLC-Chip/MS Interface (p/n G4240A) was coupled with an Agilent 6530 Accurate-Mass Q-TOF LC/MS System for LC/MS analysis.

LC parameters

HPLC-Chip

An mAb-Glyco Chip (p/n G4240-64021) was used for on-chip deglycosylation and N-glycan characterization. This chip contains a 310-nL enzyme reactor containing immobilized PNGaseF, a 160-nL enrichment column, and a 75 × 43 mm separation column, both filled with 5 µm particle size porous graphitized carbon. Note that this chip must be operated with the Chip Cube in Backflush Mode.

Flow rate

The flow rate was 3 µL/min from an Agilent 1260 capillary pump (p/n G1376A) to the enzyme reactor and enrichment column, and 500 nL/min from an Agilent 1260 nanoflow LC pump (p/n G2226A) to the analytical column.

Solvents

For the capillary pump, Agilent mAb-Glyco Deglycosylation Buffer (p/n G4240-64023) plus 3 % acetonitrile (A). For the nanoflow pump, 0.1 % formic acid in water (A); 90 % acetonitrile with water with 0.1 % formic acid (B).

Sample loading

Agilent 1260 Capillary Pump at 100 % A.

Amount of sample injected onto chip

Methods are optimized for a 2 µL injection volume. Recommended 2 µL of a 0.5 mg/mL antibody dilution for a strong glycan signal (resulting in approximately 75 ng antibody on-column).

Sample analysis

Gradient with an Agilent 1260 Nanoflow LC Pump as shown in Table 1.

MS parameters

Spectra were recorded in positive ion and both centroid and profile mode. VCap: 1,860 V and a drying gas flow of 6 L/min at 325 °C was used.

Fragmentor voltage

The fragmentor voltage was 120 V. Data were acquired at 2 GHz, for MS only mode, the range was 100–3,000 m/z at 1 spectra/sec. An internal mass calibration sample was infused continuously during the LC/MS runs. The internal reference mass system allows for accurate, automated mass calibration correction during LC/MS runs.

Data analysis

The data obtained from LC/MS were analyzed using features contained in the following software packages: Agilent MassHunter Qualitative Analysis, PCDL Manager, Agilent MassHunter Profinder, and Agilent Mass Profiler Professional.

Molecular feature extraction

The raw data were processed using the Find by Molecular Feature algorithm called Molecular Feature Extractor (MFE) within MassHunter Qualitative Analysis software. Extracted molecular features were processed to create a list of glycan compounds. These compounds were identified by searching an accurate mass library of 144 of the most common N-linked glycans found on all monoclonal antibodies created using PCDL Manager and supplied with the mAb-glyco chip.

Profinder

Following feature extraction and glycan ID, data were saved as a .cef file and imported into MassHunter Profinder for targeted recursive feature finding.

Mass Profiler Professional

After data curation using MassHunter Profinder, data were saved as a .cef file and imported into Mass Profiler Professional (MPP) for differential analysis.

Table 1. Pump gradient.

Time (min)	B (%)
Initial	3
6	3
7.5	32
8	85
9	85
9.01	3

Table 2. Timetable of chip cube valve position (100 μ M seat capillary).

Time (min)	Function	Parameter
0	Change inner valve position	Inner valve position: Analysis
0	Change outer valve position	Outer valve position: Bypass
1	Change inner valve position	Inner valve position: Analysis
1	Change outer valve position	Outer valve position: Inline
1.2	Change inner valve position	Inner valve position: Analysis
1.2	Change outer valve position	Outer valve position: Bypass
5	Change inner valve position	Inner valve position: Enrichment
5	Change outer valve position	Outer valve position: Inline
6	Change inner valve position	Inner valve position: Analysis

Results and Discussion

Glycan profiling

The goal of our study was to compare the glycan population of antibodies generated from different production methods to ascertain the reason for differential binding by ELISA. The glycan populations of four antibodies were compared. Antibodies 3.2 and 3.7 were produced from hybridoma cells grown *in vitro*. Antibodies 11 and 13 were produced from the same hybridoma cells injected into mice, producing ascites fluid from which the antibodies were harvested. All antibodies were diluted to 0.5 mg/mL and injected on the Agilent mAb-Glyco chip for in-line deglycosylation and subsequent MS analysis of liberated sugars¹. There were five technical replicates for each of the four mAb samples. The data generated from MS analysis of the antibodies were subjected to automated data analysis, similar to the workflow reported previously¹. The Q-TOF data files were processed using MassHunter Qualitative Analysis and MassHunter Profinder Software packages. Compounds were first extracted using the Find By Molecular Feature algorithm MFE. MFE allows for unbiased grouping of all related ion signals into a single compound. The compounds were subsequently annotated by searching a database supplied with the mAb-Glyco chip. Figure 1 shows results from MassHunter Qualitative Analysis after glycans were extracted using MFE and annotated using a PCDL database search.

The molecular features of each glycan compound were then re-extracted using targeted feature extraction in MassHunter Profinder. In addition to reprocessing of the data, MassHunter Profinder allowed for the observation of spectral and chromatographic peak extraction and verification of the mass and time alignment. Spectra and chromatograms for each glycan compound were reviewed for quality as well as proper retention time and mass alignment. Any glycans that were not extracted consistently across samples were manually extracted. Figure 2 illustrates the results of a single

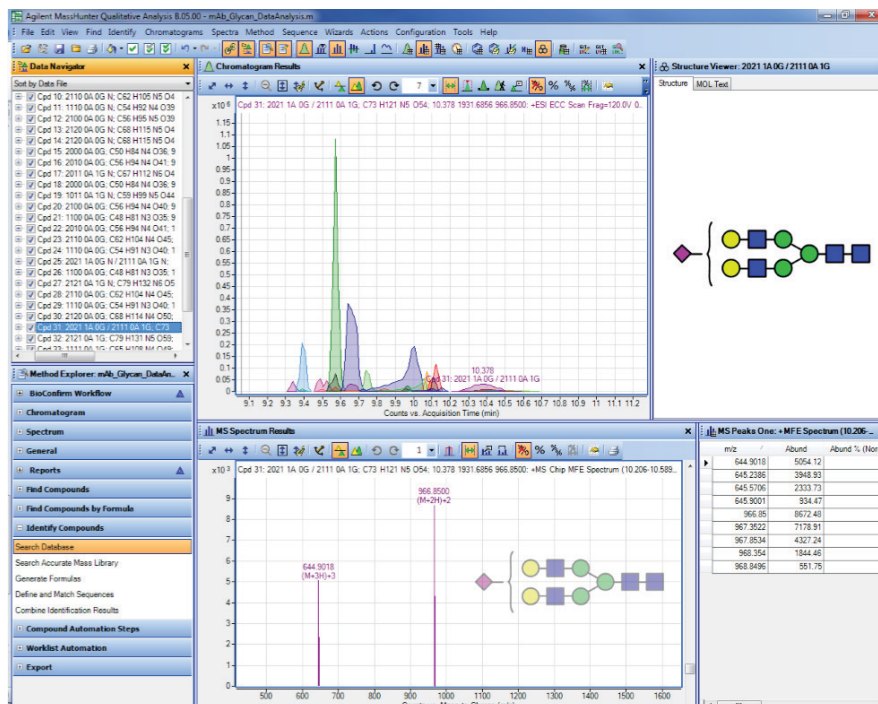


Figure 1. Agilent MassHunter Qualitative Analysis MFE.

compound. The chromatograms and spectra for this glycan are shown for all nine data files. These data were then exported as .cef files for further analysis using MPP.

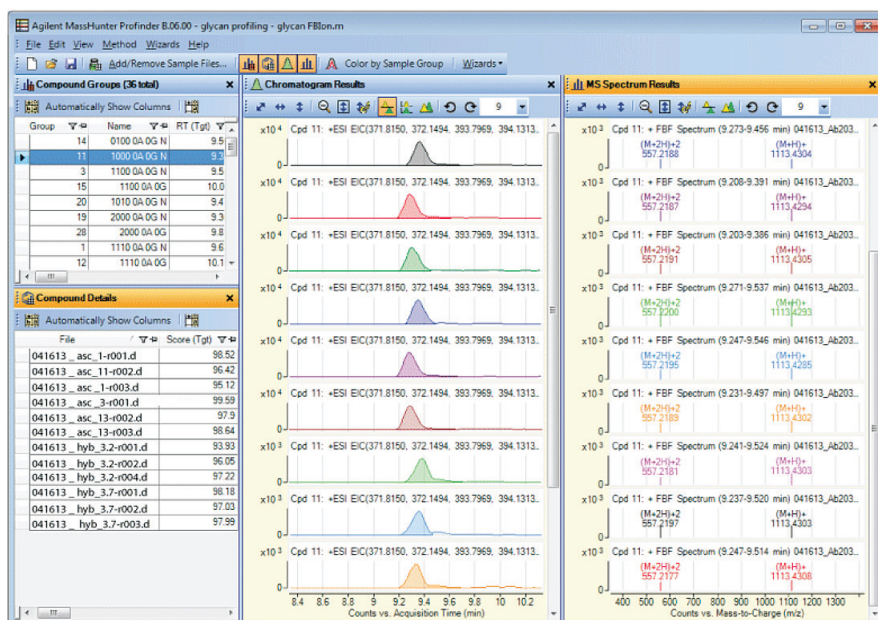


Figure 2. Agilent MassHunter Profinder.

Agilent Mass Profiler Professional (MPP)

MPP Software permits MS-based differential analysis and determination of relationships among different samples or groups². The mAb glycan data were imported and samples were grouped based on specific antibodies (3.2, 3.7, 11, and 13) and the source (hybridoma versus ascites). In total, 36 glycan species were included in the analysis.

Principal components analysis (PCA)

A PCA can be used to describe the variance within a dataset. A PCA transforms datasets of high dimensionality to just a few dimensions called principal components. Transforming the data to principal components allows observation of patterns within a dataset, and relationships between samples. The first principal component, plotted on the x-axis, often describes the feature that contributes the most to the variance within the dataset. Figure 3 shows the results of a PCA of the glycan data. The majority of the variance (52 %) is described in the first principal component on the x-axis where two primary clusters are evident. These clusters correspond to antibody source, hybridoma or ascites. In addition, within these primary clusters, the specific antibody preparations also segregate and are separated on the y-axis, principal component 2 (~21 %). These PCA results indicate that the glycan profiles differ greatest according to the source of the antibody, and to a lesser although clearly discernible extent, according to the specific antibody preparation.

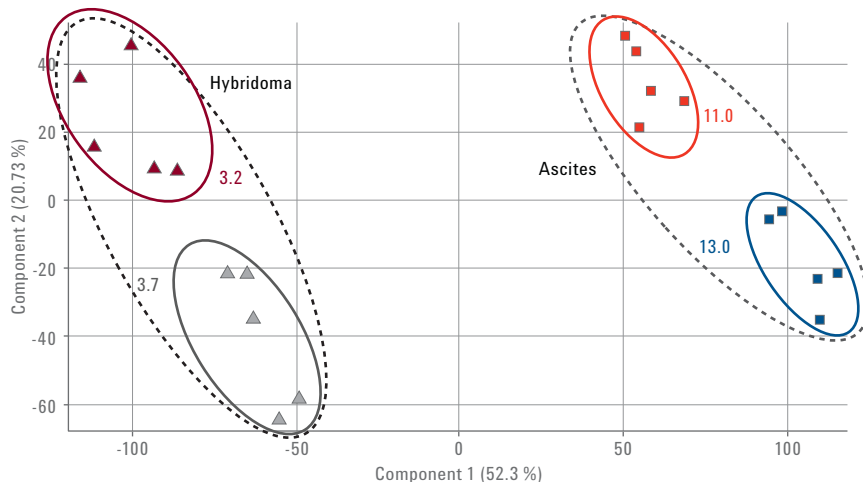


Figure 3. PCA plot of the glycan data.

Correlation-covariance (C-C) plot

One can further mine the data by looking at the C-C plot generated from the PCA. In this plot, each glycan is plotted based on the correlation score (the reliability of the relationship between the glycans) and the covariance score (the amount of change for each glycan observed across the samples). The C-C plot generated from the PCA analysis of the glycan datasets is shown in Figure 4. Glycans in the upper right corner of the plot have a high correlation score (~0.8) but have a low covariance score (close to 0). Thus, these glycans show a small change in

absolute abundance between production conditions, but the change is consistent across all of the samples within each set of antibodies. This can be seen in the abundance profile for these specific glycans shown in inset A. Conversely, the glycans in the lower left corner of the plot have high covariance scores because their abundance profiles differ greatly between samples, most notably between ascites and hybridoma antibodies (inset B). These glycans also have high correlation scores and their abundance profiles are very similar across samples.

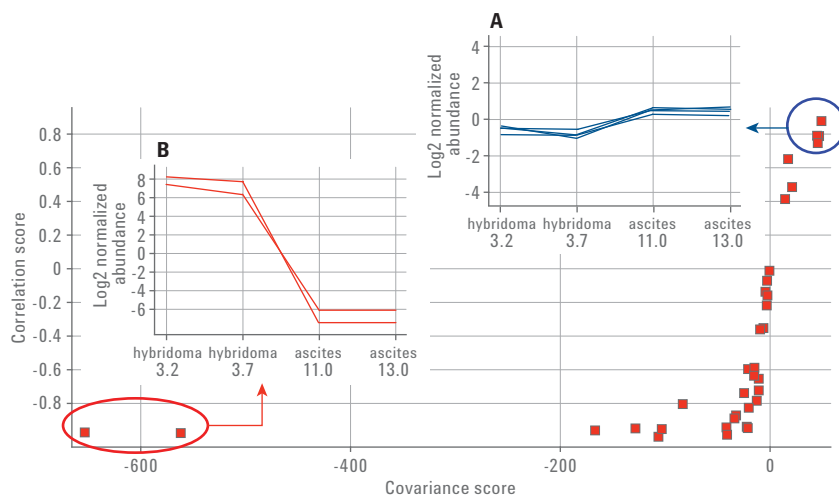


Figure 4. C-C plot of the glycan data.

In addition, we examined and confirmed the contribution of individual sugars to the overall sample variance through the individual glycan profiles as illustrated in Figures 5 and 6. In Figure 5, the $(\text{Hex})_4(\text{HexNAc})_3(\text{dHex})_1(\text{NeuGc})_1$ located at the top of the plot (close to 0) is tightly correlated and has little covariance among samples. Looking at the raw counts plotted in a bar graph for each technical replicate, as well as each separate antibody sample, one

can see very little variability is observed across technical replicates, and only an approximate 2-fold change is observed between antibody production types. Thus, the amount of this glycan is slightly elevated in the ascites preparations. This is anticipated based upon the location of this data point on the C-C plot. In contrast, Figure 6 highlights a high mannose glycan $((\text{Hex})_8(\text{HexNAc})_2 \text{ amine})$, that is located in the bottom left of the plot, away from the origin, which shows

tight correlation among sample replicates and large covariance across sample sets. Looking at the raw counts plotted in a bar graph, there is very little variability observed across technical replicates; however, this sugar appears to be present only in the antibody samples produced in hybridoma. Thus, the C-C plot is a good way to see the segregation of data based on the compound variance.

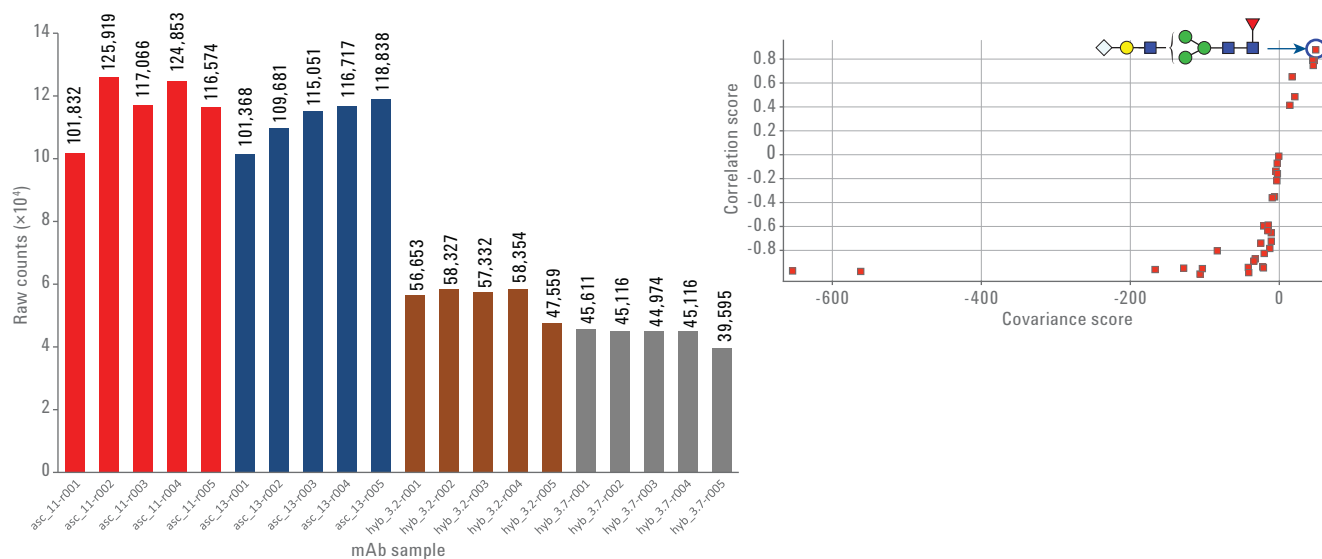


Figure 5. $(\text{Hex})_4(\text{HexNAc})_3(\text{dHex})_1(\text{NeuGc})_1$ glycan with high correlation and low covariance.

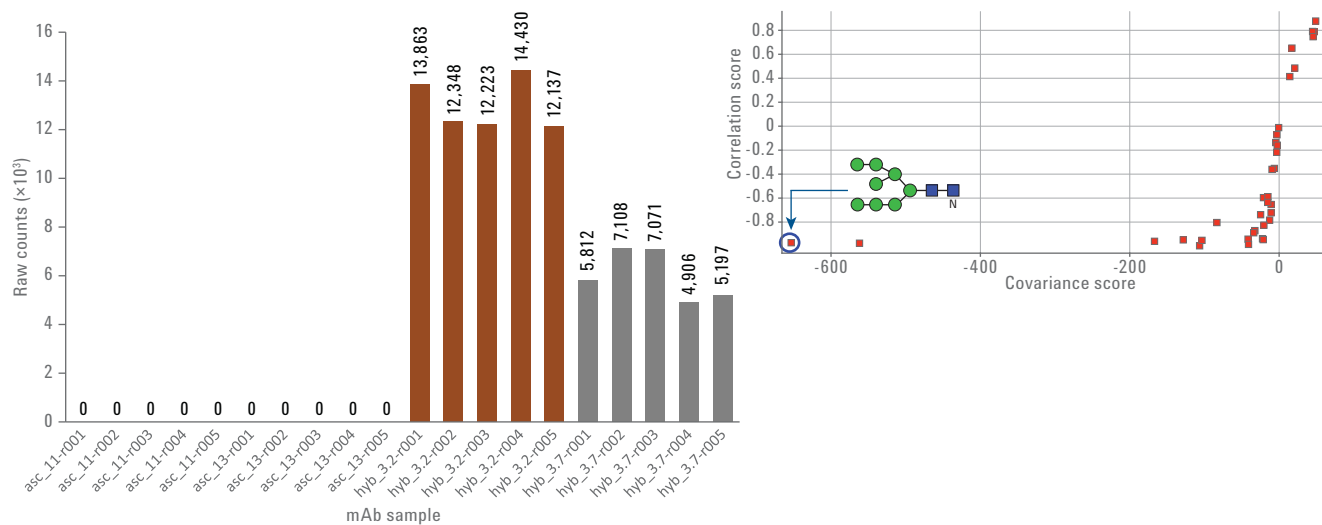


Figure 6. $(\text{Hex})_4(\text{HexNAc})_3(\text{dHex})_1(\text{NeuGc})_1$: glycan with high correlation and low covariance.

Distribution of glycans across antibody preparations

To determine which glycans are significantly different between antibodies, an ANOVA was performed. Of the 36 glycans in the dataset, 26 pass an ANOVA filter using a p -value of 0.05. Of these 26 glycans, 21 had average abundance values at least 1.5-fold difference between at least two antibody types. The majority of these differences were between ascites and hybridoma. Most of the observed glycans were present in all antibodies. The Venn diagram in Figure 7 was used to determine if any glycans were unique to any of the antibodies. Only two glycans, $(\text{Hex})_7(\text{HexNAc})_4(\text{dHex})_1$ and $(\text{Hex})_8(\text{HexNAc})_2$ amine, were unique to hybridoma-derived antibodies. We can use similar approaches to look at sugars that vary among hybridoma or ascites samples, highlight inter-replicate differences, and analyze the contributions of sample handling to variance (data not shown). One goal of this study was to determine if glycan population differences between antibodies would provide insight into how the ELISA binding is being affected by the sugars present on the Fc portion of the antibody. Our hope was that we could simply attribute charge differences to the differential binding observed in the ELISA study. One of the differentially expressed glycans, $(\text{Hex})_4(\text{HexNAc})_3(\text{dHex})_1(\text{NeuGC})_1$, does contain a sialic acid which is charged; however, this glycan is present in all antibodies, and is only slightly elevated in the ascites. The two glycans that are unique to hybridoma are not in the top five glycans based on abundance. Thus, it is difficult to attribute functional differences to a single unique glycan or the overall glycan population in this case.

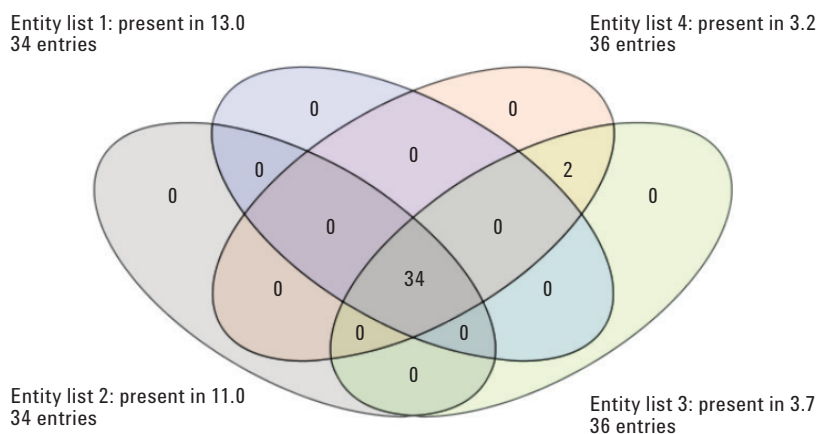


Figure 7. Venn diagram of glycan population.

Conclusions

The Agilent suite of software allows for high resolution, high accuracy MS, automatic and recursive feature extraction, database searching with comprehensive and customizable databases, and powerful differential analysis. Although this specific example demonstrates the use of the technology with a glycan dataset, this workflow is applicable to any situation where related biological samples collected under different experimental conditions are being compared. For our study, we were able to find a subset of glycans that were unique to a single source of antibody production. Unfortunately, those glycans do not lend themselves to a simple interpretation for differential binding, and further experimentation is necessary to explain the mechanistic differences between these antibodies.

References

1. Trojer, L., Gromadski, K., Buckenmaier, S. The Agilent mAb-Glyco Chip Kit for rapid and fully automated characterization of N-linked glycans from monoclonal antibodies, *Agilent Technologies Technical Overview*, publication number 5990-6924EN, **2011**.
2. Agilent G3835AA MassHunter Mass Profiler Professional Software, *Agilent Technologies Familiarization Guide*, publication number G3835-90010 Rev. A, **2012**.

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