The Agilent mAb-Glyco Chip Kit for rapid and fully automated characterization of N-linked glycans from monoclonal antibodies

Technical Overview

Introduction

Monoclonal antibodies (mAbs) represent an important class within the wide range of new biological entities with about 30 antibody drugs licensed for the treatment of various diseases\(^1\). These glycoprotein pharmaceuticals bear complex oligosaccharide moieties within their structure, whose presence, absence and profile can have significant impact on therapeutic efficacy, pharmacokinetics, immunogenicity, folding and stability. Glycosylation can be influenced by many factors such as the cell line in which the mAb is produced as well as specific production conditions including pH, temperature and medium. Thus, the characterization of glycan profiles is of vital importance throughout the various phases of therapeutic mAbs development.

Traditional PNGase F based characterization of N-glycans typically takes between one half and two days to complete, including deglycosylation, hydrolysis, derivatization of glycans, glycan analysis and data processing. In contrast, the Agilent mAb-Glyco Chip Kit (G4240-64020), designed to characterize N-linked glycans, is an automated workflow solution that delivers results in 10 to 30 minutes. The analyst simply dilutes and centrifuges the mAb sample and runs it on the Agilent 1260 Infinity HPLC-Chip/MS system. mAb deglycosylation, glycan separation and direct transmission of the analytes to MS-detection occurs on-chip. Data processing is comprehensive, requires very little hands-on time, and can typically be completed in less than 5 minutes per sample providing the analyst with fast results. Thus, the Agilent mAb-Glyco Chip removes a major bottleneck during the development phase of these biological drugs.

This Technical Overview describes:

- the instrumental setup of the Agilent 1260 Infinity HPLC-Chip/MS system
- the design of the Agilent mAb-Glyco Chip Kit
- the automated N-glycan characterization analysis and data processing workflow
- the stability, reproducibility and lifetime data of the Agilent mAb-Glyco Chip.
**Instrument Setup**

All analyses described herein were performed using an Agilent 1260 Infinity HPLC-Chip/MS system consisting of a micro autosampler (G1377A) with sample thermostat (G1330B), capillary pump (G1376A), nanoflow pump (G2225A) with micro degasser (G1379B), and a Chip-Cube (G4240A) that interfaces LC modules and the MS instrument. HPLC-grade H₂O [0.1% FA] and ACN [0.1% FA] were used as nanoflow pump mobile phases A and B, respectively. The capillary pump (sample loading) was operated in the isocratic mode with the deglycosylation buffer (G4240-64023), installed on channel A. Mass detection occurred with an Agilent 6520 Accurate-Mass Q-TOF (G6520B). Internal mass calibration used m/z 922.0098 from the API-TOF Reference Mass Solution Kit (G1969-85001). The mAb-Glyco Chip Enablement Kit (G4240-64025) must be installed prior to use of the mAb-Glyco Chip ensuring chip robustness and maximum lifetime. MassHunter Workstation was used for data acquisition and processing (LC/MS Data Acquisition (B 02.01) and Qualitative Analysis (B 03.01) software).

**Agilent mAb-Glyco Chip Kit**

The kit incorporates the mAb-Glyco Chip, the Reagent Pack, the mAb-Glyco Chip Content Disk, and a Quick Start Guide. The reagent pack provides all chemicals needed for ready chip operation: System Conditioning Reagent for flow path deactivation and carryover minimization; Glycan Standards for chromatographic checkout and method development; Antibody Standard for functional checkout and troubleshooting; and Deglycosylation Buffer for dilution of standards, samples and for loading the mAb samples onto the chip’s enzyme reactor. The Content Disk has optimized methods for HPLC-Chip/MS analysis and data processing, including efficiency tools such as glycan accurate mass and structure database for ease of characterization, and reporting templates. More details can be found in the mAb-Glyco Chip User’s Guide².

**Agilent mAb-Glyco Chip Layout**

Figure 1 illustrates the architecture of the mAb-Glyco Chip. It integrates: (a) a 310-nL enzyme reactor (ER), packed with immobilized PNGase F beads, (b) a 160-nL porous graphitized carbon enrichment column (PGC-EC), (c) a 43-mm PGC separation column (PGC-SC), and the nano electrospray tip (not shown) for direct transfer of analytes to MS-detection. The mAb-Glyco Chip uses the concentric rotor-in-rotor valve design of the Chip-Cube³, which allows for switching ER and PGC-EC independently into or out of the capillary pump flow path. Table 1 summarizes all possible chip valve positions.

<table>
<thead>
<tr>
<th>Inner Rotor (IR)</th>
<th>Outer Rotor (OR)</th>
<th>Columns Nanoflow Pump</th>
<th>Columns Capillary Pump</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enrichment</td>
<td>Inline</td>
<td>PGC-SC</td>
<td>ER / PGC-EC</td>
</tr>
<tr>
<td>Analysis</td>
<td>Inline</td>
<td>PGC-EC / PGC-SC</td>
<td>ER</td>
</tr>
<tr>
<td>Analysis</td>
<td>Bypass</td>
<td>PGC-EC / PGC-SC</td>
<td>None</td>
</tr>
</tbody>
</table>

Table 1

Possible rotor valve switching positions of the Agilent mAb-Glyco Chip.
Description of the On-Chip Workflow

1) Data Acquisition

Figure 2 illustrates the on-chip workflow comprising five automated steps:

1. **Sample injection** (Figure 2A): The outer rotor is set to bypass, the inner rotor to analysis. A volume of antibody sample, which is approximately 10 times larger than the ER-volume, is injected and loaded onto the chip using the capillary pump. Note in Figure 2A that there is no column in the loading flow path. Sample injection merely serves to transport the sample to the correct position for subsequent enzyme reactor fill.

2. **Enzyme reactor fill** (Figure 2B): The outer rotor switches to the inline position for a defined period of time, which correlates with capillary pump flow rate and the ER-volume. This step introduces the sample to the on-chip workflow by loop injection, where the ER cuts a piece of the sample from the heart of the injected plug by time-based outer rotor valve switching.

Figure 2
Valve switching scheme of the mAb-Glyco Chip for automated on-chip deglycosylation of mAbs and subsequent on-chip enrichment, separation and MS based detection of cleaved N-glycans. Note that the mAb-Glyco Chip has to be operated in the backflush mode (more details in Reference 2).

(Continued)
3. **Deglycosylation** (Figure 2C): The outer rotor switches back to bypass. The sample in the ER is allowed to interact with the PNGase F enzyme for a user-defined period of time, while the capillary pump flushes the system.

4. **Glycan transfer** (Figure 2D): Both the inner and outer rotors turn at the same time switching both ER and PGC-EC into the capillary pump flow path. The cleaved N-glycans are transferred from the ER to the PGC-EC where they become trapped.

---

3. **Deglycosylation** (Figure 2C): The outer rotor switches back to bypass. The sample in the ER is allowed to interact with the PNGase F enzyme for a user-defined period of time, while the capillary pump flushes the system.

4. **Glycan transfer** (Figure 2D): Both the inner and outer rotors turn at the same time switching both ER and PGC-EC into the capillary pump flow path. The cleaved N-glycans are transferred from the ER to the PGC-EC where they become trapped.

---

**Figure 2**

Valve switching scheme of the mAb-Glyco Chip for automated on-chip deglycosylation of mAbs and subsequent on-chip enrichment, separation and MS based detection of cleaved N-glycans. Note that the mAb-Glyco Chip has to be operated in the backflush mode (more details in Reference 2). (Continued)
5. Glycan separation/detection
(Figure 2E): The inner rotor turns back into analysis position so that both the PGC-EC and PGC-SC are in the nanopump flow path. A regular reversed phase gradient elutes the trapped N-glycans onto the PGC-SC where chromatographic separation occurs prior to transmission of the analytes to MS-detection. During analysis, the outer rotor keeps the inline position for cleaning and re-equilibration of the ER with deglycosylation buffer.

Figure 2
Valve switching scheme of the mAb-Glyco Chip for automated on-chip deglycosylation of mAbs and subsequent on-chip enrichment, separation and MS based detection of cleaved N-glycans. Note that the mAb-Glyco Chip has to be operated in the backflush mode (more details in Reference 2).
Figure 3 shows results obtained from the analysis of the antibody standard using method “Antibody_75µmSeat Capillary.m:”, which comes as part of the mAb-Glyco Chip Kit. The top pane shows time segments of the workflow, the capillary pressure trace, and the nanopump gradient. At a capillary pump flow rate of 3 µL/min, the sample injection (segment A) requires 1 min and the ER fill (B) 6 sec. Reaction time (C) is 4 min, which typically results in complete deglycosylation of antibodies. The transfer of cleaved N-glycans from the ER to the PGC-EC (D) takes 1 min and the separation of the enriched glycans on the PGC-SC (E) including a column flushing step and re-equilibration, required 6 min. This makes a total of 12 min for a workflow that traditionally could take from one-half to several days.

The bottom pane shows the corresponding chromatogram. The red peaks are glycans that result from mAb deglycosylation and the black peaks are the internal standard that is spiked into the antibody standard. The internal standard is a free reducing end glycan that serves for functional checkout of the HPLC-Chip/MS system. For instance, on an intact mAb-Glyco Chip the ratio between the most abundant antibody glycans and the internal standard should be similar to that shown in Figure 3.

Figure 3
Analysis speed of the mAb-Glyco Chip. The whole workflow time, including sample injection (A), enzyme reactor fill (B), deglycosylation (C), glycan transfer (D) and glycan separation/detection (E) is 12 min. Sample: antibody standard from the mAb-Glyco Chip reagent pack, 75 ng on-column.
Taking a closer look at the glycan chromatogram of the antibody standard, which is shown in Figure 4 including structure assignments. Why is the same glycan structure assigned to three distinct peaks? Figure 4 illustrates that during enzymatic deglycosylation by PNGase F, glycosylamines are released from the polypeptide backbone of the antibody. These are reactive intermediates that hydrolyze under acidic conditions to free reducing end glycans. Due to the anomeric equilibrium at the carbon on the reducing end, each glycosylamine has two corresponding free glycan structures. The chromatogram on the right shows that these diastereomeric species can be resolved on the PGC separation column so that each cleaved N-glycan results in three peaks. Since the workflow with the mAb-Glyco Chip occurs within such a short time frame (minutes), the predominately detected species are glycosylamines. Yet, exposure to the acidic nanoflow pump gradient (0.1% FA) converts some amount of glycosylamine to free reducing end glycans (between 5% to 10%). For low abundant glycosylamine structures identified (orange peaks) the free reducing end glycans are below the limit of detection.

*The peaks for the internal standard were removed for reasons of simplicity.

---

**Figure 4**
Left: Chemistry of the enzymatic cleavage of N-linked glycans from a polypeptide backbone by PNGase F. The glycosylamine intermediate hydrolyzes into two anomic free reducing end glycan species. Right: Due to rapid workflow the mAb-Glyco Chip predominately detects glycosylamines. Sample: antibody standard from the mAb-Glyco Chip reagent pack, 75 ng on-column. (Internal standard not extracted for reasons of simplicity.)
2) Automated Data Analysis

Data obtained from the analysis of N-glycans cleaved off mAbs can be complex (broad distribution of glycans, glycosylamines and corresponding free reducing end glycan for each species (Figure 3)). Therefore an automated, comprehensive data analysis and reporting procedure was developed to facilitate interpretation. Figure 5 provides an overview of the steps that convert a raw data file (TIC) to a report of identified glycans with attached information on structure, retention time, volume, mass error and relative abundance.

**Extraction:** The Q-TOF data file is processed using the Molecular Feature Extractor (MFE) that is part of the MassHunter Qualitative Analysis software. MFE uses an algorithm that considers LC retention time and accurate mass to extract unique compounds from the TIC data set. Furthermore, comparisons are made between the compound masses detected and those stored in the accurate mass mAb-Glyco database.

**Identification (optional):** By searching the extracted glycan signals against the mAb-Glyco database, structures are attached to the hits. Figure 6 shows MassHunter Qual results obtained from the antibody standard. The identified glycan hits are listed in the Data Navigator pane and corresponding peaks are superimposed in the Chromatogram Results (overlay of Extracted Compound Chromatograms (ECC’s)). The Structure Viewer illustrates the glycan structure of a selected peak. m/z values, charge states and isotopes of the selected glycan hit are displayed in MS Spectrum Results and MS Spectrum Peak List.

**Reporting:** Volumes of glycosylamines with identical mass are merged. The same occurs for the volumes of the free reducing end glycan isomers. The latter are multiplied with an ionization factor that compensates for the difference in ionization efficiency between glycosylamines and free reducing end glycans. Corresponding glycosylamines and free glycans are clustered and the total volume is reported. Volumes of free reducing end glycan entries are deleted as redundant information. Finally, glycosylamine isomers are illustrated by color coding.

![Figure 5](image-url)  
*Figure 5*  
Schematic representation of the automated glycan extraction, identification, clustering and reporting procedure.
Figure 6
Glycan results of an extraction (MFE) and identification (Search Database) procedure with MassHunter Qualitative Analysis Software. Sample: antibody standard from the mAb-Glyco Chip reagent pack, 75 ng on-column.
Chip Stability, Reproducibility and Lifetime

Table 2 summarizes characteristics of the mAb-Glyco Chip. Retention time stability over 200 injections was in the range of 0.3% to 0.5% RSD (four chips evaluated). Reproducibility of the relative abundance was typically better 5% RSD for glycans > 1% relative ratio. Spray stability was comparable to that of standard HPLC-chips. Typical lifetime was in the range of 200–300 injections (10 chips evaluated). Under appropriate storage conditions (~20 °C, wet ER), the immobilized PNGase F conserved 90% of its original activity after a period of three months. Multiple freeze/thaw cycles do not severely affect PNGase F activity after immobilization.

Figure 7 (A): Glycan chromatograms were obtained from injection 1 and 200. Retention time, relative glycan distribution and absolute signal intensities of the identified glycan pattern remain comparable, demonstrating full catalytic activity of the PNGase F reactor over the course of 200 injections.

Figure 7 (B): %RSD values for relative glycan ratios in the range of 1.6% to 3.9% show robustness and the stability of the fast on-chip deglycosylation workflow.

Table 2
Overview of characteristics of the mAb-Glyco Chip.

<table>
<thead>
<tr>
<th>Investigated Item</th>
<th>Number of Chips tested</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention time stability over 200 injections</td>
<td>4</td>
<td>0.3% to 0.5% RSD</td>
</tr>
<tr>
<td>Intra-day reproducibility of rel glycan ratios</td>
<td>2</td>
<td>5% RSD on average (glycans &gt; 1% relative ratio)</td>
</tr>
<tr>
<td>Inter-day reproducibility of rel glycan ratios</td>
<td>2</td>
<td>7% RSD on average (glycans &gt; 1% relative ratio)</td>
</tr>
<tr>
<td>Spray stability</td>
<td>1</td>
<td>&gt; 200 hours</td>
</tr>
<tr>
<td>Chip lifetime</td>
<td>10</td>
<td>200-300 injections (75 ng IgG from bovine serum (Sigma) on-column each injection)</td>
</tr>
<tr>
<td>PNGase F activity // long-term storage</td>
<td>1</td>
<td>90% remaining activity after 3 months of storage at –20°C</td>
</tr>
<tr>
<td>PNGase F activity // freeze/thaw cycles</td>
<td>2</td>
<td>83% remaining activity after 74 freeze/thaw cycles</td>
</tr>
</tbody>
</table>

(A)
Injection No. 1
Non-fucosylated glycosylamines
Fucosylated glycosylamines
Sialylated glycosylamines
Free reducing end glycans

(B)
Injection No. 200

Figure 7
Long-term stability and robustness of the mAb-Glyco Chip: (A) Extracted glycan pattern of the analysed antibody at injection number 1 and 200; (B) Relative glycan ratio as function of number of injections performed (only 4 most intense N-glycans considered). Sample: IgG from bovine serum (Sigma), 75 ng on-column.
**Conclusion**

This note provides a technical description of the new mAb-Glyco Chip Kit that was designed for fast and automated characterization of N-glycans from monoclonal antibodies (mAb) with the Agilent 1260 Infinity HPLC-Chip/MS system. It is demonstrated that the complete workflow including on-chip deglycosylation of the mAb as well as enrichment, chromatographic separation, and Q-TOF detection of the cleaved glycans, and data processing can be completed in about 15 min. Furthermore, extensive data on mAb-Glyco Chip stability, reproducibility and lifetime are shown. It is concluded that the mAb-Glyco Chip Kit provides a robust turnkey solution that helps to remove a major bottleneck during the development phase of mAb-based biological drugs allowing the analyst to quickly provide answers.

**References**


2. mAb-Glyco Chip User’s Guide (G4240-90020); provided with mAb-Glyco Chip Enablement Kit (softcopy on mAb-Glyco Chip Content Disk).

3. “Agilent 1100 Series HPLC-Chip/MS system,” Agilent Technologies publication 5989-3627EN.
