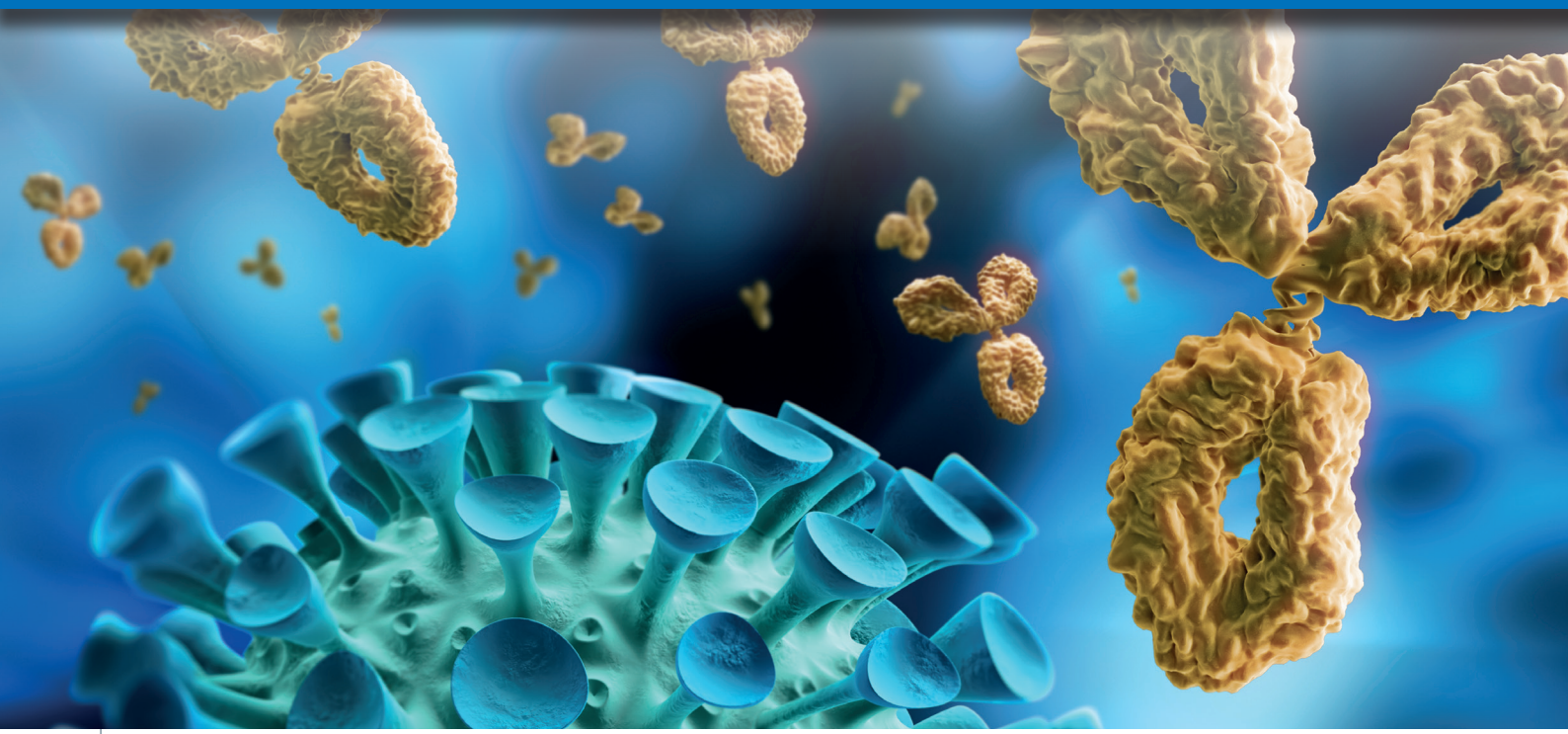

Automated High-Throughput Clone Screening of Therapeutic Antibodies: From Automated IdeS Digestion to Intact Fc/2-Glycosylation Analysis by MALDI-TOF

The glycosylation status of the Fc/2 region is a critical quality attribute (CQA) of therapeutic antibodies and is typically tested in early stages of development to select the best clones.

Abstract

High-throughput methods are needed to screen multiple clones which include all steps from preparing the free Fc/2 and making it available for MS analysis. The established method includes an IgG1 digestion using IdeS, which cleaves the mAb into the Fc/2 and the F(ab´)2 domains. Subsequent MALDI analysis works best with the DHAP/DAC matrix, which can be challenging to prepare manually. Here, the entire process from digestion to MALDI sample preparation was established using laboratory automation followed by standardized acquisition and data processing methods.

Keywords: High-Throughput Screening, autoflex MALDI-TOF, 2,5-DHAP, IdeS, Fc/2, mAb, Glycosylation, clone screening

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This improved protocol adjusts all steps of the method for use on a robotic platform. MALDI is an ideal match for enzymatic screening protocols and is already established in high-throughput primary screens for drug development (1) and enzyme activity screening (2). In contrast to common ESI methods, LC separation is not required, which accelerates the entire process providing a throughput of approximately 2x384 samples per day, based on use of 96 well MTPs.

Introduction

MALDI-TOF mass spectrometry is a robust and easy-to-use approach to characterize biologics even without LC purification. However, sample preparation used to depend on users' skills and experience and was, therefore, less popular in the industry. Automated robot preparation protocols can overcome these obstacles providing reliable and reproducible results while giving scientists time to focus on higher value activities.

The MALDI matrix 2,5-dihydoxyacetophenone (DHAP) is ideal for the analysis of proteins as it provides highest peak resolution and sensitivity (3). The formation of abundant 2+ and 3+ ions using DHAP further enhances protein analysis.

However, the DHAP sample preparation involves a seeded crystallization step in the sample vial to initialize analyte and matrix co-crystallization before they are spotted to the MALDI sample plate. In the manual procedure, crystallization is initiated by repeated up-and-down pipetting and scratching the pipet tip along the inner wall of the vial.

This is a challenge for robotic automation. We developed an automated protocol by adding glass beads to the matrix-analyte mixture and have the pipetting robot shake them at high frequency until crystallization is initiated.

This protocol can be used on most robotic platforms. It produces the free Fc/2 subunit from the intact mAb for glycan profiling and prepares the samples on the MALDI sample plate, ready for MALDI-TOF analysis at a throughput of 2x384 samples per day.

Experimental

Preparation of solutions and PCR plate for the liquid handler automation

1. Preparation of DAC solution: Dissolve 18 mg of DAC in 1000 µL of deionized water, vortex and spin down.

2. Preparation of 2,5-DHAP solution: Dissolve 20 mg of 2,5-DHAP in 1000 µL ethanol, vortex and spin down. For further information, please refer to the Bruker Instructions for Use for DHAP.

Table 1: List of materials used

Material	Order Information/Details
BIOSPEC glass beads	Zirconia/Silica Beads (Cat. No. 11079105z 0.5mm dia)
PCR Plate (96 wells)	Eppendorf twin.tec® PCR Plate 96, # 0030 128.648
IgG Sample	NISTmAb reference material RM 8671 (NIST)
IdeS Enzyme	FabRICATOR, (Genovis # AO-FRI-096)
TFA	Trifluoroacetic acid (Merck #1.08178.0050)
Ethanol	Ethanol HPLC grade (Riedel-de Haen #34852)
DAC	Diammonium citrate (Merck #247561)
MALDI Matrix DHAP	2,5-DHAP (2,5-Dihydroxyactetophenone), (Bruker # 8231829)
MALDI Mass Calibrant	Protein Mix I, (Bruker # 8206355)
MALDI Sample Plate	MTP AnchorChip 384 (800μm), (Bruker # 8280790)
Automated Liquid Handling Station	Bravo Automated Liquid Handling Platform (Agilent), with Shaking/Heating Stations
MALDI-TOF MS	autoflex maX (Bruker)
BPC	BioPharma Compass 2021 (Bruker)

3. Preparation of 2,5-DHAP/DAC matrix solution: 375 µL 2,5-DHAP solution (20 mg/mL) and 125 µL of DAC solution (18 mg/mL) were added to a vial (Eppendorf), the solution was briefly vortexed, sonicated (15 min@RT) and centrifuged to provide a clear matrix solution. The supernatant was transferred to a 1.5 mL vial for matrix solution.

   Note:The matrix solution can be kept @RT in the dark for approx. 5 days until color changes from white to yellow, then make fresh.

4. Antibody solution (1 µg/µL): Intact NISTmAb (10 mg/mL) was diluted with water to 1 mg/mL

5. IdeS solution (1 u/µL): 100 units of IdeS were dissolved in 100 µL water ad 1 u/µL.

6. TFA (1%) solution: Add 1 mL of TFA into 99 mL of deionized water, vortex. WARNING: Health Hazard! Refer to the supplier's safety data sheet!

7. Loading glass beads to the PCR plate: Add between 10 and 15 beads (a spatula tip's worth of beads) per vial.

Liquid handler protocol for automatic digestion and MALDI sample preparation:

The robot was equipped with 5 96-well PCR plates preloaded with either IdeS solution, antibody solution, TFA (1%) solution, matrix solution and glass beads. The IdeS plate - for digestion - and the glass beads plate - for MALDI preparation - were placed on the heating station and shaking station, respectively.

1. Digestion: Add 10 µL of the antibody solution and 10 µL of the IdeS solution, incubate 30 min, @37°C in the heating station (open lids).

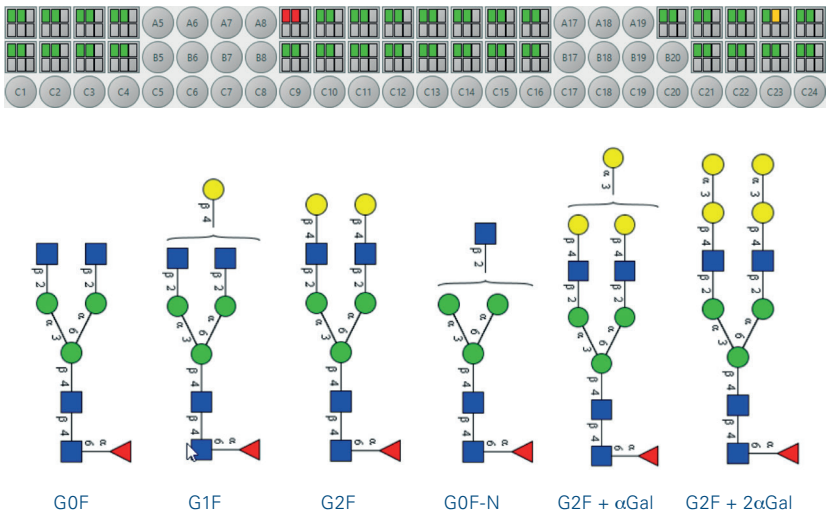

Figure 1: (Top) Status overview of analyzed MALDI spectra in BioPharma Compass, displaying the status of 2 attributes for 32 replicates of the NIST Fc/2 subunit plus 1 negative control. The traffic light report reflects the success of the analysis in comparison to expected results. A more detailed report of the quality attributes of position B2 is shown in Figure 4. (Bottom) Six glycoforms of the NIST Fc/2 subunit were detected using this method.

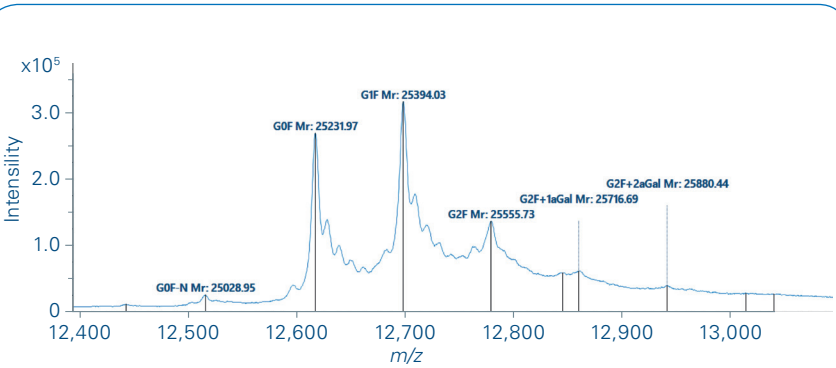

Figure 2: MALDI-TOF spectrum of 2+ ions of NIST Fc/2 (position B2), with the 6 glycoforms annotated in BioPharma Compass

Annotation	Mr Ref	Δ Mr [ppm]	Δ Mr [Da]	Rel. Int. Ref [%]	Rel. Int. Sample [%]	Confirmed
-	23786.7			5.0	0.0	No
G0-N	24866.7			1.0	0.0	No
G0F-N	25028.8	6.20	0.2	3.0	3.1	Yes
G0	25085.8			6.0	0.0	No
G0F	25232.0	0.46	0.0	34.7	31.7	Yes
G1F	25394.1	3.81	0.1	36.2	37.3	Yes
G2F	25556.3	21.16	0.5	7.0	16.1	Yes
G2F+1aGal	25718.4	66.70	1.7	2.0	7.2	Yes
G2F+2aGal	25880.6	4.17	0.1	5.0	4.7	Yes

Figure 3: Detailed result description for position B2, with relative intensities of the glycoforms in the reference (Rel. Int. Ref) and spectrum (Rel. Int. Sample) of glycans

2. Dilution: Add 5µL digest solution to 70 µL TFA (1%) to stop the digest and dilute the solution 1:15. The dilution step is mandatory and substitutes for further sample purification. Higher concentration of salts and buffers in antibody samples may require an even larger dilution factor.

3. MALDI sample preparation: Transfer 20 µL of the diluted sample to a vial in the PCR plate loaded with glass beads, add 10 µL of matrix solution and shake 2 min, 1500 rpm, @RT in the shaker station.

4. MALDI spotting: Transfer 1 µL (146 fmol Fc/2) of the cloudy matrix–analyte suspension to a spot on the sample holder and let dry at ambient air.

Data acquisition:

Automatically prepared NISTmAb Fc/2 samples were mass analyzed by MALDI-TOF-MS in positive linear ion mode on the autoflex maX using the default FlexControl method for the mass range from 5 to 20 kDa. The method was calibrated using the Bruker Protein Calibration Standard I prepared with the DHAP/DAC matrix in the mass range 6-17 kDa.

Table 2: Summary of the NISTmAb Fc/2 glycoform composition based on the quantitative analysis of 8 spectra. Observed coefficients of variability were in the 1-6% range

Glyco-form	Mr Ref.	Rel. Int. (%)	CV
G0F	25232.0	31.7	1.9%
G0F-N	25028.8	3.2	4.7%
G1F	25394.1	36.7	0.8%
G2F	25556.3	15.3	2.1%
G2F+1aGal	25718.4	6.9	5.6%
G2F+2aGal	25880.6	4.8	5.5%

Data analysis:

Spectra were automatically processed in FlexAnalysis using the default processing method for proteins (Proteins 5-20kDa.FAMS-Method) in the mass range 5-20 kDa and imported into BPC. For clone selection analysis based on Fc/2 glycoprofiling, the BPC workflow Multi Target Screening MALDI was used with the 2+ ions of the NISTmAb Fc/2, in the *m/z* 12,000-13,000 range.

Results

For 33 replicate samples of IdeS digested NISTmAb, MALDI-TOF spectra were acquired and analyzed in BPC using the workflow Multi Target Screening MALDI. This workflow supports the input of a sample table providing sample name, spot ID and the molecular formula of the analyte protein including a target profile with relative abundances, e.g., of glycoforms (see Figure 3: Rel Int. Ref. column). This information was retrieved from a reference sample, to compare to glycan profiles from new batches or in a clone screening experiment. The similarity between a glycan profile and the profile in the sample spectrum was the first quality attribute assessed by the cosine score (4) and translated in a color scheme: Scores higher than 0.9 (high similarity, marked in green), values lower than 0.6 (low similarity, marked in red). The medium similarity values were marked in yellow, recommending further investigation of the result. The second quality attribute was mass accuracy, which was set between 5 Da and 10 Da. In most of the spectra analyzed, mass accuracy was below 5 Da average and marked in green. At position A23 the mass error was higher (8 Da) and therefore the attribute was highlighted yellow in the status overview in Figure 1. The spectrum at position A9, marked in red, didn't contain any sample and served as a control.

For each position, annotated spectra (Figure 2) and detailed results (Figure 3) are provided in BPC 2021.

To verify the reproducibility of the screening method, a sample of n=8 spectra was analyzed and the results summarized in Table 2 and illustrated in Figure 5. The CVs for the high and lower abundant glycoforms were in the 2% and 5% range, respectively.

The automated IdeS digestion and preparation of the NISTmAb ready for MALDI-TOF analysis was implemented. The 2,5-DHAP MALDI matrix used in this approach was successfully adapted to a robotic protocol.

The method does not require any chromatographic sample workup after IdeS digestion other than the dilution step, as the Fc/2 fragment is the only digest product visible in the observed mass range (~*m/z* 13,000) - the F(ab´)2 fragment does not affect the analysis.

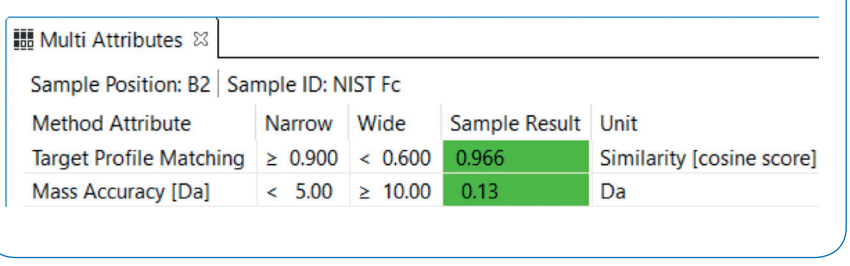

Figure 4: Legend for the attribute color codes with actual results for position B2

NIST Fc/2 spectra were automatically acquired and subsequently analyzed in BioPharma Compass. Glycoforms were automatically assigned, quantified with a reproducibility of the relative abundances better than 5% and compared to a reference glycan pattern allowing to screen for similar glycan profiles.

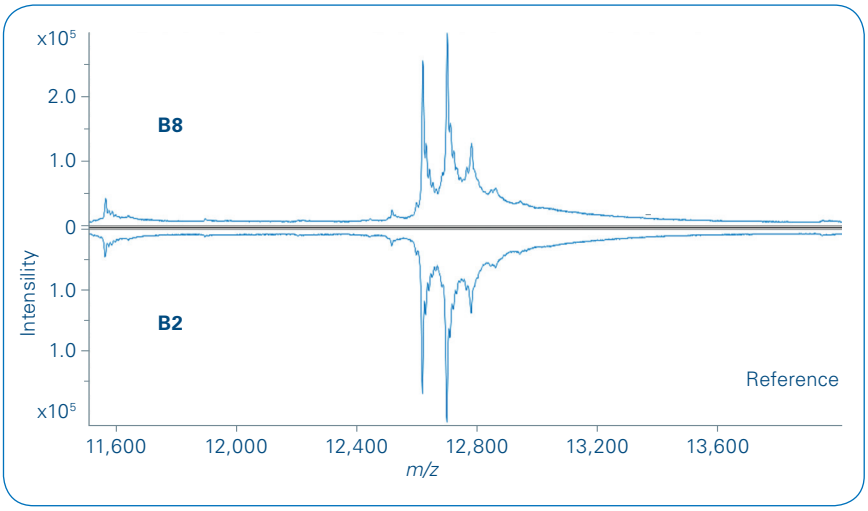

Figure 5: Butterfly plot comparing the NISTmAb Fc/2 m/z *range (z=2) of spectra from spots B2 and B8 indicates the good reproducibility of the automated screening method*

Conclusion

- The described method removes several hurdles in MALDI sample preparation allowing robust, automated high-throughput glycoform screening scenarios. This use of MALDI in LC-free scenarios provides high efficiency and reproducibility with full automation, including data analysis, enabling a throughput of 2x384 samples per day and beyond.
- The throughput of the workflow is limited by sample preparation, as MALDI spectra acquisition, processing and analysis take place in seconds. Thus, the throughput (with the described setup estimated to be 2x384 samples per day) can be further enhanced by use of 384 vial MTPs, 384 pipet heads and multiple heater/shaker stations on the robotic platform of choice. Parallelized bead addition is possible (5).
- In addition, the same, simple protocol described here can be used manually or semi automatically.

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