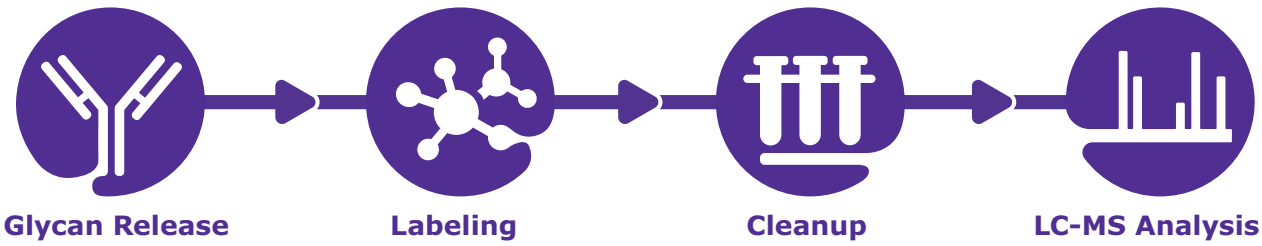
Application Note

Released N-linked Glycan Analysis Workflow of Adalimumab

Step-by-step protocol for the procainamide labeled glycan profiling of a monoclonal antibody

Romana Rigger PhD[1], Pegah Jalili[2], Uma Sreenivasan PhD[3], Kevin Ray PhD[2]
[1]Buchs, Switzerland, [2]St.Louis, MO, USA, [3]Round Rock, TX, USA

Workflow for released N-glycan analysis



A complete workflow based on UHPLC-FLR-MS is developed to analyze the N-glycan profile. The workflow offers the following:

- Step-by-step instructions for sample preparation and analysis
- Procainamide labeling for increased detection sensitivity
- Separation using BIOshell™ Glycan HPLC column
- Low flow rate for reduced solvent consumption
- Compatibility with mass spectrometry

1. Introduction

Careful and thorough characterization of therapeutic mAbs is essential to ensuring drug safety and efficacy. Hence, establishing several critical quality attributes (CQAs) for each of the proteins and demonstrating that their production batches are within acceptable limits is necessary for both the innovator and biosimilar therapeutics. In this application note, we describe glycan analysis of the innovator mAb adalimumab (Humira) as well as a recombinant mAb of the same sequence.

Monoclonal antibodies (mAbs) are target specific and have high efficacy and few side effects. Glycosylation is one of the most common and important post translational modifications for mAbs. Glycans attached to the antibodies play an important role in the pharmacokinetics, efficacy, and safety of therapeutic adalimumab. Glycosylation involves the attachment of glycans at specific sites on a protein, most commonly at asparagine (Asn, N-linked) or serine/threonine (Ser/Thr, O-linked) amino acid residues. There are two types of glycosylation— N-linked glycosylation and O-linked glycosylation, and both types are important for protein conformation, protein activity, providing protection from proteolytic degradation, and intracellular trafficking and secretion. N-glycan moieties also play a key role in the folding, processing, and secretion of proteins from the endoplasmic reticulum (ER) and the golgi apparatus. Based on the large influence of glycosylation on protein function, an accurate study and analysis of glycans is essential. Protein glycosylation is specifically mentioned in established technical guidelines, e.g., ICH Q5E and Q6B and FDA's published guidance for industry titled "Development of Therapeutic Protein Biosimilars".

There are four options to approach the N-glycan analysis: intact glycoproteins, glycopeptides, released glycans, or monosaccharides. This article focuses on the analysis of released N-glycans by UHPLC, combined with fluorescence (FLR) and mass spectrometric (MS) detection. The analysis of released N-glycans presented here is one of the most powerful and commonly used approaches for glycan composition analysis. Fluorescent derivatization increases the MS ionization efficiency and allows relative quantification of percent abundances of glycan species by fluorescence detection. Among fluorescent derivatization molecules, procainamide offers one of the best signals, surpassing the more traditional 2-AB and 2-AA labeling. Hydrophilic interaction liquid chromatography (HILIC) is a proven technique for the separation and quantitation of glycans and has notable advantages over the other HPLC separation modes (e.g. reversed-phase, anion exchange). In this protocol, a BIOshell™ Glycan HPLC column is used to analyze adalimumab N-glycans—labeled with procainamide.

Adalimumab from different sources was analyzed. It is a recombinant human IgG1 monoclonal antibody (mAb), specific for human tumor necrosis factor (TNF). It has a molecular mass of about 150 kDa and is N-glycosylated on the Fc region.

2. General procedure for released N-linked glycan analysis

2.1 Samples

Two samples are analyzed and compared using the following protocol:

- **Adalimumab Reference** (Humira®, from Abbvie, Inc., North Chicago, IL)
- **MSQC16** SILu™Lite SigmaMAb Adalimumab Monoclonal Antibody (equivalent, recombinant protein from us)

2.2 Reagent preparation

2.2.1 Buffers and Enzymes

- **8 M Guanidine HCl, prepared in water**
  1. Dissolve 7.6 g guanidine HCl in water and bring the final volume to 10 mL
  2. 0.2 mL/sample (152.85 mg guanidine HCl/sample) is required
- **50 mM Ammonium bicarbonate (ABC buffer), prepared in water**
  1. Dissolve 395 mg ABC in 100 mL water
  2. 2 mL/sample (7.91 mg ABC/sample) is required
- **1 unit/µL PNGase F**
  1. Dissolve in water; may be aliquoted and stored for 6 months or longer at -20 °C

2.2.2 System suitability reagents

The workflow, including glycan release, labeling, and SPE steps is tested on human IgG (hIgG). A sample of IgG is handled alongside other samples. Procainamide labeled dextran hydrolysate is used as an external standard for the analysis of glycans by HPLC. When analyzed on the BIOshell™ Glycan HPLC Column, the standard gives a characteristic ladder profile, from a monomeric glucose to approximately a 20-mer of glucose oligosaccharide. This ladder provides calibration reference points that can aid in identifying more complex glycans based upon retention characteristics.

- **IgG purified from human serum for workflow suitability**

  IgG purified from human serum (200 µg) is processed as a workflow suitability control.
  1. Prepare a 10 µg/µL solution in 8 M guanidine HCl and aliquot in 200 µg portions
  2. IgG is handled identically to the samples
  3. Store any unused portions at -20°C for later use
- **Dextran hydrolysate, procainamide labeled for HPLC-FLR-MS system suitability**

Dextran hydrolysate is solubilized in 25% 75 mM ammonium formate / 75% acetonitrile (v/v), and procainamide labeled according to 2.4

2.3 N-Glycan release

Prior to the analysis, samples are reconstituted to a concentration of 1 mg/mL in water and 200 µg of each protein is used for N-glycan analysis.

2.3.1 Denaturation

1. Set heating block to 50 °C
2. Start with at least 100 µL protein at neutral pH
3. Add 200 µL 8 M guanidine HCl solution and quickly vortex to mix
4. Incubate at 50 °C for 30 min to denature (shaking optional)
5. Bring the temperature of the sample to RT

2.3.2 Buffer exchange to 50 mM ABC buffer

1. Transfer sample to a 30 kDa spin filter
2. Centrifuge at 14,000 x g for 15 min
3. Add 400 µL ABC buffer
4. Centrifuge at 14,000 x g for 30 min
5. Repeat the previous two steps once more, making sure all the solution has passed through the filter
6. Discard the flow through and place filters in new collection tubes

2.3.3 Enzymatic release of glycans

1. Set up heating block to 37 °C
2. Add 50 µL of 50 mM ABC buffer to each filter unit
3. Add 4 µL of 1 UN/µL PNGase F to each filter unit
4. Cap and seal centrifuge device with parafilm
5. Incubate at 37°C for 14-20 hours with shaking at 300 rpm

Note: The digestion time can be decreased to only 30 min using **PNGase Fast** (PN# EMS0001-kit) which produces a comparable result for most antibodies.

2.3.4 Recovery of glycans

1. Centrifuge at 1,000 x g for 10 sec to collect lid condensate
2. Add 40 µL ABC buffer
3. Centrifuge at 14,000 x g for 5 min
4. Add 100 µL ABC buffer
5. Centrifuge at 14,000 x g for 5 min
6. Repeat previous two steps once more
7. Transfer glycans from collection tube to 0.6 mL microcentrifuge tubes for labeling
8. Dry the glycans using speed vacuum

2.4 Procainamide labeling

Dried samples are labeled with procainamide in a one-pot reductive amination solution, purified by normal-phase SPE, and the resulting labeled product is then dried again. Glycans are solubilized in 50 µL of 25% 75 mM ammonium formate/75% acetonitrile (v/v) prior to UPLC-FLR-MS.

Note: All preparation and labeling must be performed in a fume hood except for weighing reagents. Prepare the incubation block by moving to the fume hood and set the temperature to 65 °C.

2.4.1 Procainamide labeling reagent

1. Weigh at least 1.8 mg sodium cyanoborohydride (NaBH3CN) per labeling reaction in a tube
    a. Tare a microcentrifuge tube
    b. Transfer NaBH3CN to the tube in the fume hood; a pencil eraser-head volume is usually sufficient
    c. Cap the tube and blow off any dust with N2 gas in the fume hood
    d. Weigh the tube
2. Weigh at least 2.033 times more procainamide hydrochloride, by mass, than NaBH3CN in a separate tube
3. Prepare 9.1 µL of a 70% dimethyl sulfoxide (DMSO)/30% acetic acid (AcOH) (v/v) solution per mg procainamide
4. Solubilize the procainamide with the 70% DMSO/30% AcOH (v/v) solution
5. Ensure the solution is homogenous by vortexing
6. Add 18.5 uL of solubilized procainamide per mg of NaBH3CN

Note: NaBH3CN will not completely solubilize; As exposure to strong acid releases cyanide gas, this step especially warrants working in the fume hood

7. Add 5 µL water per mg of NaBH3CN to completely dissolve NaBH3CN
8. Cap and mix by vortexing in the fume hood to fully solubilize NaBH3CN

2.4.2. Procainamide labeling of glycans

1. Add 40 µL labeling reagent per sample to reaction solution
2. Vortex for 1 min and briefly centrifuge
3. Place capped tube in incubator block and incubate at 65 °C for 3 hours

Note: Cover with foil to limit condensation on the lid and keep dark

2.5 SPE clean up

1. Prepare glycans for clean up by adding 200 µL of 70% ACN in water solution to the labeled glycans
2. Prepare Discovery® Glycan SPE tubes and vacuum manifold
    a. Place falcon tube under cartridge for waste collection
    b. Wash with 1 mL water, with minimum pressure gradient by vacuum manifold

c. Equilibrate with 1 mL 99% ACN in water, with minimum pressure gradient by vacuum manifold

d. Stop flow when meniscus completely enters top frit

3. Loading the samples

a. Place microcentrifuge tube under cartridge for breakthrough collection

b. Add full sample volume to cartridge bed

c. Pass sample through bed by gravity

d. When meniscus completely enters top frit, add 500 µL of 99% ACN

e. Pass volume through by gravity, collecting in same tube

f. Stop flow when meniscus completely enters top frit

g. Place falcon tube under cartridge for waste collection

h. Add breakthrough + 99% ACN to bed

i. Pass volume through bed by gravity

j. Stop flow when meniscus completely enters top frit

4. Wash

a. Add 1 mL of 99% ACN, and pass the solution through cartridge with minimum pressure gradient by vacuum manifold

b. Repeat the above step four more times

5. Elute

a. Place new microcentrifuge tube under cartridge for purified glycan collection

b. Add 200 µL of 20% ACN to bed

c. Pass volume through bed by gravity

d. When meniscus completely enters top frit, repeat the previous two steps once more

e. After the collection drip has stopped, apply medium vacuum manifold pressure to evacuate all liquid from SPE to the collection tube (Total volume ~400 µL)

6. Dry glycans by SpeedVac, 2-4 h

a. Labeled glycans can be stored at -20 °C for at least 6 months

2.6 UHPLC-FLR-MS

2.6.1 Solubilize glycans

1. Dissolve the dry glycans in 50 µL of 75% ACN / 25% of 75 mM ammonium formate (v/v) pH 4.4 (adjusted with formic acid) and vortex for 2 min
2. Centrifuge at 16,000 x g for 2 min
3. Transfer 40 µL to autosampler vials

2.6.2 UHPLC-FLR-MS parameters

HPLC Parameters	
Instrument	Waters Acquity UPLC-Waters FLR fluorescence detector
Software	MassLynx 4.1
Column	BIOshell™ Glycan 15 cm x 2.1 mm, 2.7 µm
	Equilibrate the column with 20 x column volume
Column Temperature	58 °C
Column equilibration time	9 min
Sample Manager Temperature	8° C
Mobile Phase	Mobile Phase A: 75 mM Ammonium formate pH 4.4
	Mobile Phase B: Acetonitrile
Gradient	Gradient: 75% B - 59% B in 75 min
Flow	0.3 mL/mi
Injection method	Partial Loop
Injection Volume	10 µL
Run Time	110 minutes

MS Settings	
Instrument	Thermo Q Exactive™ Plus mass spectrometer
Source	IonMax
Ion source	ESI
DDA mode	One MS2 per MS, most abundant ion, any charge state
Data-Dependent Exclusion	3 s
isolation width	5 Da
Normalized Collision Energy (%)	30
Capillary (V)	4000
Capillary temperature	200 °C
Sheath gas	10
Aux gas	12
Scan Range (Da)	400-2000

2.6.3 Sample analysis

1. Run 1-2 blanks at the start of sample list
2. Analyse dextran ladder and hIgG samples first
3. After every 5 samples, run a blank followed by a dextran to update GU values
4. Run a blank after samples and before column flush
5. At completion of queue, flush column with water for 30 minutes followed by 80% ACN/20% water for 30 minutes and store

2.7 Data analysis

LC peaks are identified by their level of residue composition (**Table 1**) from the calculated glucose unit (GU) of their elution. GU levels are determined for each LC feature's retention time by interpolation to a 5th-order polynomial standard curve of the dextran hydrolysate ladder chromatogram (see **Figure 1**). Glycan assignment is done by comparing the GU values to a custom database of glycan GU values for the BIOshell™ column. The dextran ladder is analysed via UHPLC FLR-MS after every fifth sample to correct for any retention time shifts. For relative quantification, fluorescence peak areas are normalized to the sum of all identified glycan fluorescence peak areas. The limit of quantification (LOQ) is defined as 0.5% of the most abundant peak area. This allows for compositions less than 0.5% of the total peak area because composition is normalized to the sum of all peak areas. The peak area is calculated using Xcalibur Qual Browser. For general sample analysis, the Thermo Xcalibur™ Qual Browser software retrieves and records the base peak intensity for each sample.

Table 1: Glycan constituent monosaccharides.

Glycan/Modification	Short Name	Generic Name	Residue Mass	Representation
Procainamide Modification	-	-	219.17	-
N-Acetylglucosamine	GlcNAc	HexNAc	203.08	
Mannose	Man	Hex	162.05	
Galactose	Gal	Hex	162.05	
Fucose	Fuc	Fuc	146.06	
N-Acetylneuraminic Acid	Neu5Ac	Neu5Ac	291.10	

3. Results

3.1 System suitability results

The procainamide-labeled dextran hydrolysate and control acquisitions demonstrated that the UHPLC-FLR system and column were suitable to resolve and identify labeled oligosaccharides, as per the four system suitability requirements shown in **Table 2**. First the workflow, including glycan release, labeling, and SPE steps was tested with hIgG and was found to be suitable, with 14 common hIgG glycans detected—where a minimum of 10 hIgG glycan identifications are required. The second system suitability requirement was also met; peaks G1F (1,6) and G1F (1,3) were observed to be partially resolved in the fluorescence chromatogram (**Figure 1**). Although resolution between the G1F (1,6) and G1F (1,3) peaks was not complete, they could be visually differentiated upon magnification. The third system suitability requirement was met by the analysis of hIgG control. The relative abundances of a representative subset of these glycans gave an R2 correlation of 0.99 with the historical glycan profile. Finally, the slope created during analysis of the hIgG control was observed to be 0.99, which is within the slope requirement of 1.00 ± 0.07.

Table 2: Four system suitability requirements and results for hIgG.

Type	Criteria	Result
Qualitative	≥ 10 hIgG peaks detected	Yes
	G1F(1,6); G1F(1,3) Partially Resolved	Yes
Quantitative	Correlation Coefficient R2≥ 0.95	0.99
	Slope 1.00 ± 0.07	0.99

Fluorescence chromatograms of procainamide-labeled dextran hydrolysate and hIgG control.

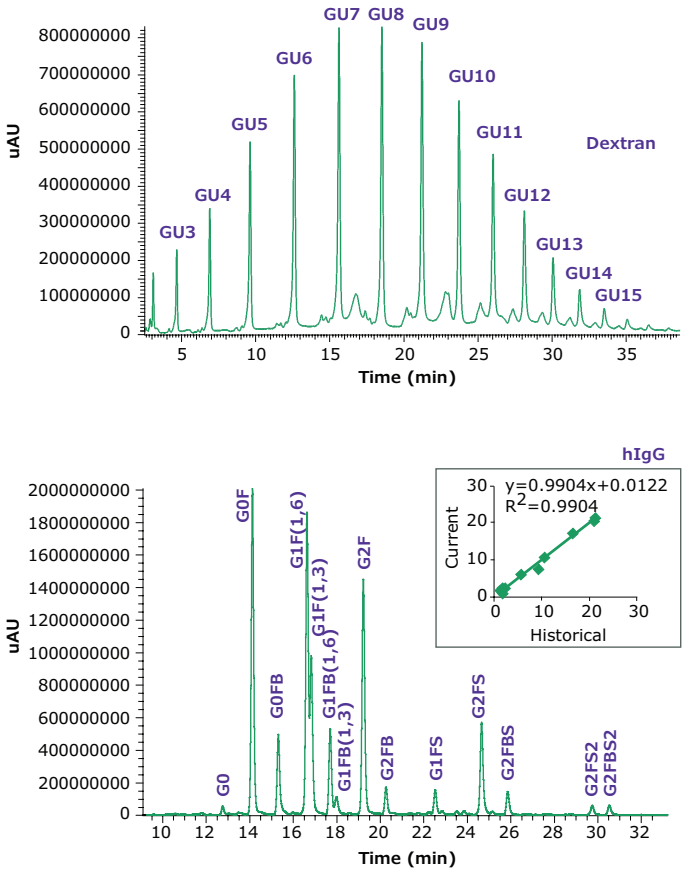


Figure 1: Fluorescence chromatograms of procainamide-labeled dextran hydrolysate and hIgG control. Top pane: annotations indicate the number of glucose units (GU) in each dextran hydrolysate-procainamide feature. Each feature of the control hIgG in the bottom pane was similarly identified. The inset shows the correlation of the glycan features' relative compositions with the historical values of previous hIgG data.

Fluorescence chromatograms of procainamide-labeled dextran hydrolysate and hIgG control showing the number of glucose units (GU) in each LC feature of the two, obtained in the released N-glycan analysis of adalimumab based on UHPLC-FLR-MS.

3.2 Adalimumab sample results

Sixteen glycan features for adalimumab samples were quantified. **Table 3** contains glycan compositions for all the samples. And **Figure 2** illustrates the fluorescence chromatograms for each sample type of adalimumab. Overall, the N-glycan profiles are broadly similar, but some differences exist in the observed relative compositions of some components. **Table 4** illustrates the structures of the observed glycans. The area under the peaks of glycans in the TIC chromatogram are summed and MS spectra were created to confirm the mass of glycans. **Figure 3** shows typical examples of MS spectra.

Table 3: N-Glycan % Composition.

Peak	Glycan	Adalimumab Reference (%)	MSQC16 (%)
1	G0-N	0.6	0.5
2	G0F-N	4.1	0.6
3	G0	0.9	3.1
4	Man5	5.3	1.0
5	G0F	65.0	44.4
6	G1F-N	1.7	-
7a	G1(1,6)	0.4	1.1
7b	G1(1,3)	0.4	0.7
8	Man6	1.8	-
9a	G1F(1,6)	12.1	29.6
9b	G1F(1,3)	5.1	10.0
10	G2F	2.7	7.3
11	G1FS(1,3)	-	0.5
12a	G2FS(1,6)	-	0.6
12b	G2FS(1,3)	-	0.4
13	G2FS2	-	0.4

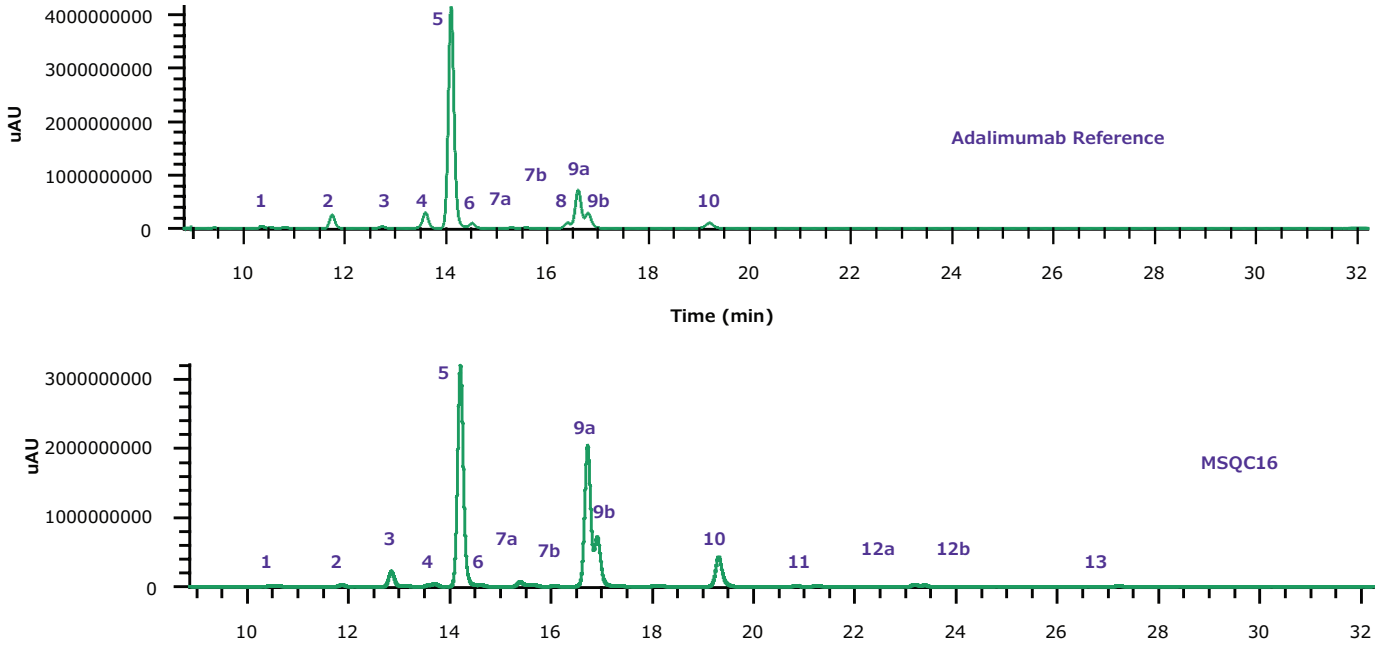


Figure 2: Fluorescence chromatograms of adalimumab samples with the numbered LC features obtained in the released N-glycan analysis of adalimumab based on UHPLC-FLR-MS.

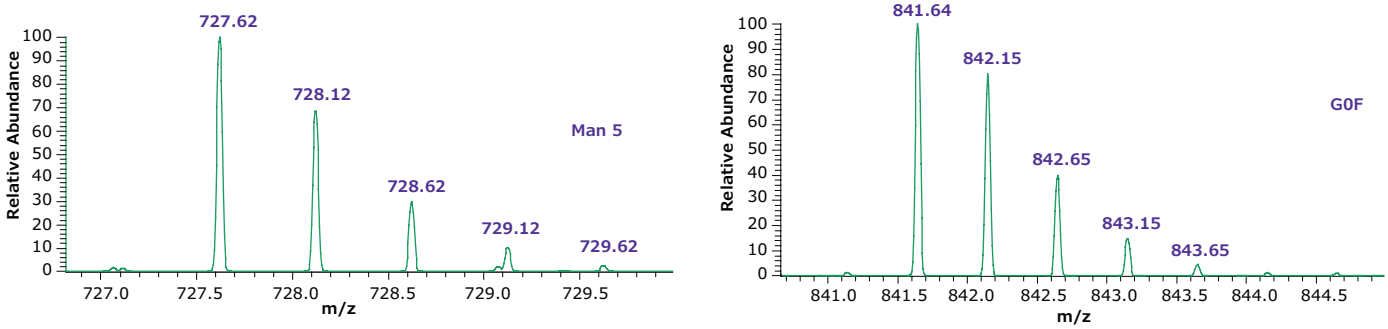


Figure 3: MS spectra of peak 4 (Man 5) and 5 (G0F) obtained on the fluorescence chromatograms of adalimumab samples in the released N-glycan analysis of adalimumab based on UHPLC-FLR-MS.

Table 4: N-Glycans Observed.

Peak	RT (min)	RT GU (min)	Theoretical Mass	Glycan	Monosaccaride Composition	Structure
1	10.46	5.29	1332.58	G0-N	Man3 GlcNAc3	
2	11.83	5.75	1478.64	G0F-N	Fuc1 Man3 GlcNAc3	
3	12.80	6.07	1535.66	G0	Man3 GlcNAc4	
4	13.66	6.35	1453.61	Man5	Man5 GlcNAc2	
5	14.17	6.51	1681.72	G0F	Fuc1 Man3 GlcNAc4	
6	14.56	6.65	1640.69	G1F-N	Fuc1 Man3 Gal1 GlcNAc3	
7a,	15.36	6.91	1697.71	G1	Man3 Gal1 GlcNAc4	
7b	15.62	6.99				
8	16.44	7.28	1615.66	Man6	Man6 GlcNAc2	
9a,	16.68	7.35	1843.77	G1F	Fuc1 Man3 Gal1 GlcNAc4	
9b	16.87	7.42				
10	19.27	8.26	2005.82	G2F	Fuc1 Man3 Gal2 GlcNAc4	
11	21.09	8.94	2134.9	G1FS	Fuc1 Man3 Gal1 GlcNAc4 Neu5Ac1	
12a,	23.23	9.78	2296.92	G2FS	Fuc1 Man3 Gal2 GlcNAc4 Neu5Ac1	
12b	23.47	9.88				
13	27.23	11.56	2588.01	G2FS2	Fuc1 Man3 Gal2 GlcNAc4 Neu5Ac2	

4. Conclusion

A complete UHPLC-FLR-MS workflow has been developed to simplify the analysis of N-linked glycans. This workflow offers the following:

- MS and Fluorescence compatibility
- System suitability testing using human IgG
- Rapid and reproducible N-Glycan release. The protocol provides detailed instructions for sample washing and denaturation
- Procainamide labeling ensuring high fluorescence intensity and ESI efficiency while showing comparable chromatographic separation compared to the other fluorescence labeling systems
- BIOshell™ HPLC column based UHPLC-FLR-MS analysis— suitable for the analysis of protein-linked glycans— and typical mobile phases used for hydrophilic interaction liquid chromatography (HILIC)
- Complete listing of all reagents, consumables, and related products.

A total of 16 glycan features were quantified for the mAb adalimumab. The glycan profile, including the qualitative and quantitative aspects, is comparable to the results found by other analytical laboratories.[1, 2]

Characterizing and monitoring the glycosylation pattern of therapeutic mAbs is required by regulatory authorities to ensure efficacy and safety of the drug.

This detailed protocol can be used for the analysis of N-linked glycans of mAbs and for complex and heterogenous glycoproteins.

References

1. Nayoung Lee, JongAh Joanne Lee, Hahymn Yang, Serim Baek, Soohwan Kim, Sooshin Kim, Tongkeun Lee, Dami Song & Gwangmin Park (2019) Evaluation of similar quality attribute characteristics in SB5 and reference product of adalimumab, mAbs, 11:1, 129-144, DOI: 10.1080/19420862.2018.1530920
2. Tebbey PW, Varga A, Naill M, Clewell J, Venema J. Consistency of quality attributes for the glycosylated monoclonal antibody Humira® (adalimumab). MAbs. 2015;7(5):805-811. doi:10.1080/19420862.2015. 1073429

Product List

Product	Cat. No.
Samples and System Suitability Reagents	
SILu™Lite SigmaMAb Adalimumab Monoclonal Antibody	**MSQC16**
Dextran from Leuconostoc mesenteroides	**31417**
IgG from human serum	**I4506**
Glycan Release	
Guanidine hydrochloride	**50933**
Ammonium bicarbonate	**09830**
PNGase F from Elizabethkingia meningoseptica	**F8435**
Microcon®-30kDa Centrifugal Filter Unit with Ultracel-30 membrane	**MRCF0R030**
Labeling	
Sodium cyanoborohydride	**156159**
Procainamide hydrochloride	**PHR1252**
Dimethyl sulfoxide	**D8418**
Acetic acid	**695092**
Cleanup	
Discovery® Glycan SPE Tube	**55465-U**
Acetonitrile	**1.00029**
VM20 Vacuum Manifold	**VM20**
HPLC	
BIOshellTM Glycan HPLC Column, 15 cm x 2.1 mm, 2.7 µm	**50994-U**
Acetonitrile	**1.00029**
Ammonium formate	**70221**
Formic acid	**5.33002**
Water	
Ultrapure water from Milli-Q® system e.g. Milli-Q® IQ 7000	**ZIQ7000T0**
Water for UHPLC-MS LiChrosolv®	**1.03728**
Accessories	
Microcentrifuge tubes volume 0.6 mL	**T5149**
Autosampler vials volume 0.3 mL	**29661-U**

Supelco® products Sigma-Aldrich® products Milli-Q® products Millipore® products

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64293 Darmstadt, Germany

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