TRASTUZUMAB GLYCAN BATCH-TO-BATCH PROFILING USING A UPLC/FLR/QTOF MS PLATFORM

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INTRODUCTION

- Glycosylation plays a vital role in the safety and efficacy of many therapeutic proteins such as recombinant monoclonal antibody (rmAb).
- Several studies have shown the correlation between glycosylation variations caused by cell line selection and changes in culture medium parameters.[1] These variations can have profound effect on the biological activity of the rmAb.
- Therefore, regulatory agencies ask drug manufactures to supply detailed assessment of the glycan micro-heterogeneity and batch-to-batch consistency.
- In this study, we applied a robust, sensitive and reproducible analytical platform that comprises a Ultra Performance Liquid Chromatography (UPLC), a Fluorescent (FLR) detector and a Xevo QTof Mass Spectrometry (MS) for batch-to-batch glycan profiling of *Trastuzumab*, a widely used rmAb drug for breast cancer treatment.
- Our results showed that the three batches of Trastuzumab have different glycan profiles quantitively and qualitatively. Relative quantitation of the 2AB-labeled glycans was calculated using the intergraded FLR chromatographic peak area.

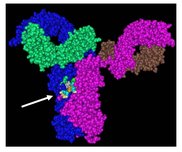


Figure 1: Crystal structure of Trastuzumab. The arrow points to the oligosaccharides that are located in the Fc region on the heavy chain.

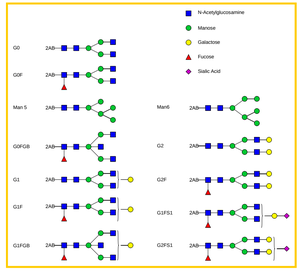


Figure 2: A list of common IgG glycans.

METHODS

1. Sample Preparation

- Trastuzumab was solubilized in 0.1% RapiGest and 50 mM Ammonium Bicarbonate, and was reduced with Dithiothreitol and alkylated with Iodoacetamide and deglycosylated with PNGase F overnight.

2. HILIC µElution Plate for Glycan Extraction

MassPREP™ HILIC µElution™ plate

Pre-condition wells
200 µL 100% H_2O

Condition wells
200 µL 90% ACN (x2)

Reconstitute samples
in 90 % ACN

Load samples into wells
in 90% ACN

Wash sample loaded wells
With 500 µL 90% ACN (x2)

Elute glycans from wells
with 100 µL 1mM
Ammonium Tris-Citrate in10% ACN (x2)

3. FL-labeling with 2-aminobenzamide (2AB)[2]

4. Remove excess 2AB reagent (same as Step 2.)

- The eluted glycans was lyophilized and reconstituted in 40 µl of 50% Acetonitrile prior to UPLC/FLR/MS analysis.

5. UPLC/FLR/Xevo QTof MS

LC conditions

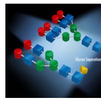
LC System:	Waters® ACQUITY UPLC® System
Column:	ACQUITY UPLC BEH Glycan Column 2.1 x 150 mm, 1.7 µm
Column Temp:	40 °C
Flow Rate:	400 µL/min.
Mobile Phase A:	100 mM Ammonium Formate, pH 4.5
Mobile Phase B:	Acetonitrile
Gradient:	72-62% B in 45 min.
Weak Wash:	75% Acetonitrile
Strong Wash:	20% Acetonitrile
Injection:	5.0 µL partial loop

FLR conditions

FLR:	Waters® ACQUITY UPLC® Fluorescence detector
Excitation:	330 nm
Emission:	420 nm
Data Rate:	1 pts/sec
PMT Gain:	1.00
Time Constant:	Normal

MS conditions

MS System:	Waters Xevo™ QTof MS
Ionization Mode:	ESI +
Capillary Voltage:	3200 V
Cone Voltage:	35 V
Desolvation Temp:	350 °C
Desolvation Gas:	800 L/Hr
Source Temp:	120 °C
Acquisition Range:	800 —2000 m/z
Collision Energies:	6 V
Lock Mass:	CSI (1 µg/µl in 50% Isopropanol)



RESULTS

1. UPLC/FLR Injection-to-Injection Reproducibility

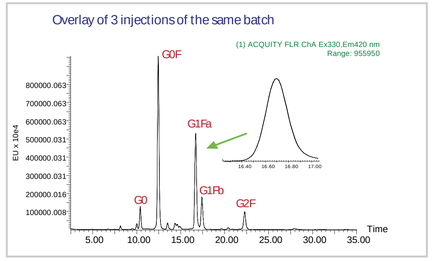


Figure 3: UPLC/FLR chromatograms of three injections. The analytes were 2AB-labeled glycans released from one Trastuzumab batch. The retention times for all peaks were overlaid perfectly. The peak area response variation was less than 2% (RSV%). The most abundant glycans were labeled here.

2. Xevo-QTof MS for Mass Profiling

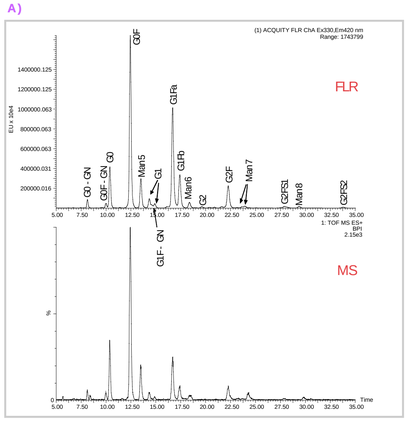


Figure 4. A) UPLC/FLR/Xevo QTof MS analysis of 2AB-labeled glycans. The amount of glycan injected was released from 12.5 µg of rmAb. The glycan identities were confirmed by their m/z values; some glycans were subjected to MSMS fragmentations. The mass error is less than 30 ppm. B) Spectra of Extracted Ion Chromatogram (XIC) of G2S1 and G2S2. C) Spectra of XIC of Man5, Man6, Man7 and Man8; Man7 exists in isomeric forms.

3. Batch-to-Batch Glycan Profiling Results

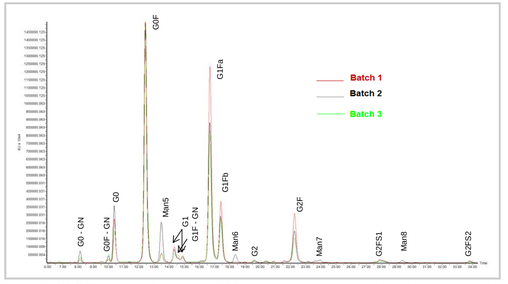


Figure 5. UPLC/FLR chromatograms of 2AB-labeled glycans released from three different Trastuzumab batches. The chromatograms were overlaid. The glycan profile looked different among the batches. For example, batch 2 has significantly more High Mannose glycans.

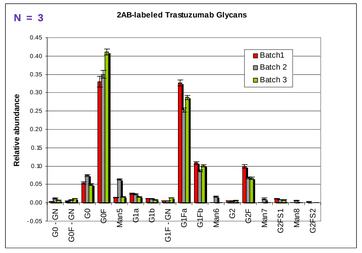


Chart 1. Plot of the relative concentration of 2AB-labeled glycans from three batches of Trastuzumab. Triplicate samples (N=3) were analyzed; which meant that three aliquots of the deglycosylated Trastuzumab were subjected to µElution cleanup and FL labeling and the 2nd µElution cleanup to remove excess 2AB.

Table 1. Summary data table .

	Batch 1		Batch 2		Batch 3	
	Rela. Conc. (%)	RSD (%)	Rela. Conc. (%)	RSD (%)	Rela. Conc. (%)	RSD (%)
G0 - GN	0.34 ± 0.01	3.78	1.22 ± 0.14	11.50	0.71 ± 0.01	1.69
G0F - GN	0.36 ± 0.04	11.96	0.74 ± 0.09	11.94	1.19 ± 0.16	13.00
G0	5.35 ± 0.22	4.14	7.43 ± 0.28	3.86	4.95 ± 0.35	7.10
G0F	33.03 ± 0.85	2.58	34.90 ± 1.1	3.11	41.09 ± 1.49	3.63
Man5	1.41 ± 0.05	3.80	6.35 ± 0.11	1.75	1.61 ± 0.02	1.03
G1a	2.45 ± 0.04	1.70	2.25 ± 0.15	6.62	1.61 ± 0.15	9.00
G1b	1.20 ± 0.06	5.04	1.06 ± 0.03	2.98	0.77 ± 0.02	2.81
G1F - GN	0.55 ± 0.003	0.49	0.55 ± 0.02	3.16	1.38 ± 0.04	2.64
G1Fa	32.65 ± 0.59	1.80	25.22 ± 0.62	2.44	28.72 ± 0.77	2.68
G1Fb	10.83 ± 0.30	2.75	8.68 ± 0.2	2.25	10.06 ± 0.44	4.37
Man6	—	—	1.68 ± 0.13	7.89	—	—
G2	0.54 ± 0.04	7.55	0.42 ± 0.04	10.03	0.59 ± 0.03	5.10
G2F	9.89 ± 0.31	3.10	6.82 ± 0.23	3.44	6.63 ± 0.47	7.14
Man7	—	—	1.08 ± 0.17	16.11	—	—
G2FS1	1.09 ± 0.08	7.62	0.79 ± 0.02	2.59	0.71 ± 0.03	3.55
Man8	—	—	0.55 ± 0.07	12.90		
G2FS2	0.31 ± 0.04	14.31	0.26 ± 0.05	18.20	—	—

CONCLUSION

- Waters Glycan Characterization Platform, UPLC/FLR/Xevo QTof MS, offers a robust and sensitive analytical solution for batch-to-batch glycan profiling.
- UPLC HILIC separation of FL labeled glycans showed highly resolved glycan peaks.[3] The separation was very reproducible; which enabled accurate glycan assignment and quantitation.
- Sample preparation for FL labeling on released glycans can take up to two days to accomplish, it is often viewed as laborious and time consuming. However, we demonstrated that our optimized sample preparation procedure was very robust and reproducible and easy to follow. The purpose of analyzing FL-tag glycans was to utilize the sensitive FL detection.
- Xevo QTof MS offered sensitive and accurate mass detection; therefore, the combination of UPLC retention time and the highly accurate m/z values from QTof MS improves glycan assignments.

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References

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2. *Bigge JC, Patel TP, Bruce JA, Goulding PN, Charles SM, Parekh RB. "Nonselective and efficient fluorescent labeling of glycans using 2- amino benzamide and anthranilic acid." Anal Biochem 230: 229-238, 1995.*
3. *Joomi Ahn, Jonathan Bones, Ying Qing Yu, Pauline Rudd, Martin Gilar. "Separation of 2-aminobenzamide labeled glycans using hydrophilic interaction chromatography columns packed with 1.7 µm sorbent". J. Chrom. B. 878 (2010) 403–408.*

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