A Single Analytical Platform for Glycan Analysis, Charge Heterogeneity, and Purity Determination of the NISTmAb

Fast Methods to Maximize the Use of the PA 800 Plus

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Glycan microheterogeneity, charge heterogeneity, and product-related impurities can all threaten the safety, stability, and efficacy of monoclonal antibodies (mAbs) and mAb-based therapeutics. As such, the comprehensive characterization of mAb therapeutics is necessary during manufacturing and storage. However, no single analytical technique is sufficient. A comprehensive characterization traditionally requires multiple analytical tools and techniques, consuming both time and resources. In addition, traditional techniques often require labor-intensive and time-consuming sample preparation.

The work outlined in this note demonstrates a considerable advancement in the analysis of mAbs. A single analytical platform, the capillary electrophoresis-based SCIEX PA 800 Plus Pharmaceutical Analysis System, was used to successfully assess the N-glycan microheterogeneity, charge heterogeneity and purity of the National Institute of Standards and Technology monoclonal antibody (NISTmAb) reference material (RM 8671). The SCIEX EZ-CE cartridge and SCIEX Fast Glycan Technology simplified and streamlined the workflows, allowing results to be achieved faster (Figure 1 & 2).

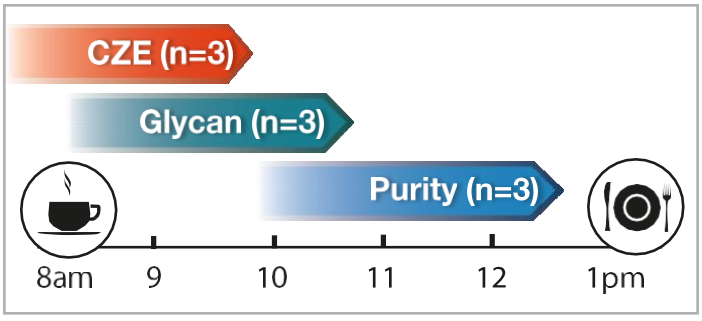

Figure 1. Flexible workflows on the PA 800 Plus help you accomplish a lot before noon.

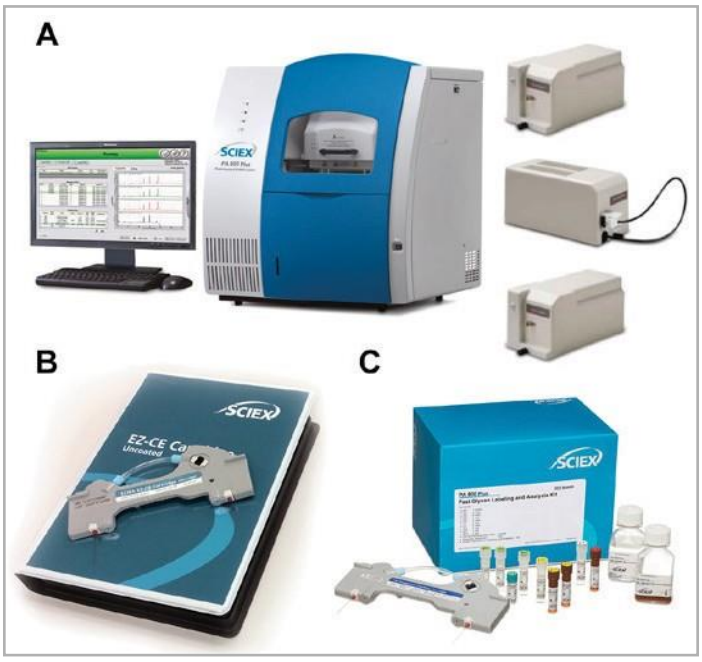

Figure 2. A. PA 800 Plus Pharmaceutical Analysis System B. EZ-CE Cartridge C. Fast Glycan Technology

Key Feature of the PA 800 Plus

- The PA 800 Plus is a capillary electrophoresis-based analytical platform with the flexibility to characterize mAb N-glycan microheterogeneity, charge heterogeneity, and purity
- The Fast Glycan Technology vastly simplifies N-glycan sample preparation, speeds up analysis, and facilitates immediate glycan identification
- The ready-to-use EZ-CE cartridge provides a one-stop, universal cartridge for all analyses

Methods

Glycan Analysis CE with laser-induced fluorescence detection (CE-LIF) on the PA 800 Plus was used to determine the N-linked glycan profile of the NISTmAb. Sample preparation was accomplished in just 60 minutes using the Fast Glycan Technology with the workflow outlined in Figure 3. Briefly, the N-linked glycans were rapidly cleaved using PNGase F with an on-bead digestion at 60°C. Cleaved glycans were labeled with the charged fluorophore, aminopyrene trisulfonate (APTS), at 60° C. Excess dye was removed and the sample was eluted with water. The released glycans were separated and detected in just 5 minutes using the PA 800 Plus and ready-to-use EZ-CE cartridge. Further details can be found in Guttman et al.[1]

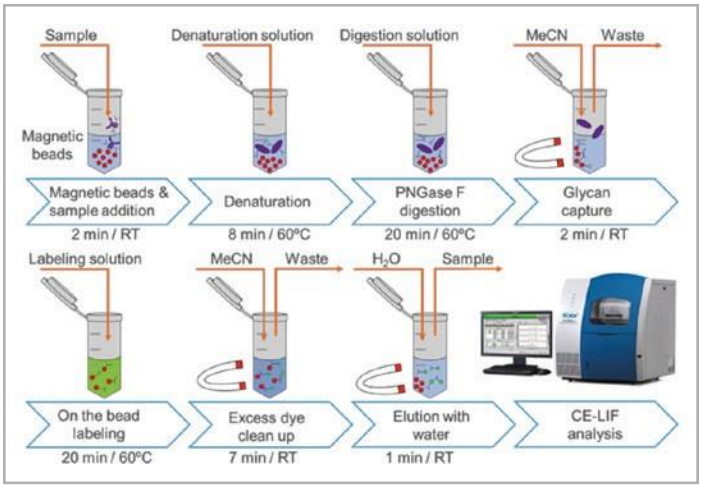

Figure 3. Workflow for the Fast Glycan Technology.

Rapid Charge Variant Profiling Traditionally, CZE of mAbs has employed a self-assembled cartridge with a 40 cm effective length. For this analysis, the CZE conditions described previously by Santos[2] were employed, minor modifications were made to adapt the method to the 20 cm effective length of the EZ-CE cartridge. The separation voltage was reduced to 12 kV and cartridge temperature was reduced to 20° C.

Purity Determination The sample preparation protocols outlined in the PA 800 Plus Application Guide were followed for both reduced and non-reduced assays.[3] Sample was injected on the short side (10 cm effective length) of the EZ-CE cartridge. Methods have been described previously by Gallegos-Perez.[4]

Glycan Analysis

The optimized CE-LIF analysis using the PA 800 Plus and EZ-CE cartridge produced a high-resolution separation in just 5 minutes. The electrophoretic peaks were automatically assigned GU values and identified. Figure 4A highlights the most abundant glycans of the NISTmAb. Figure 4B, a zoomed view, reveals at least 26 identified glycan species that were present over a wide range of abundances. The 26 identified glycans are listed in Table 1 along with their relative abundances. Peak assignments were confirmed using a serial exoglycosidase digestion.[5]

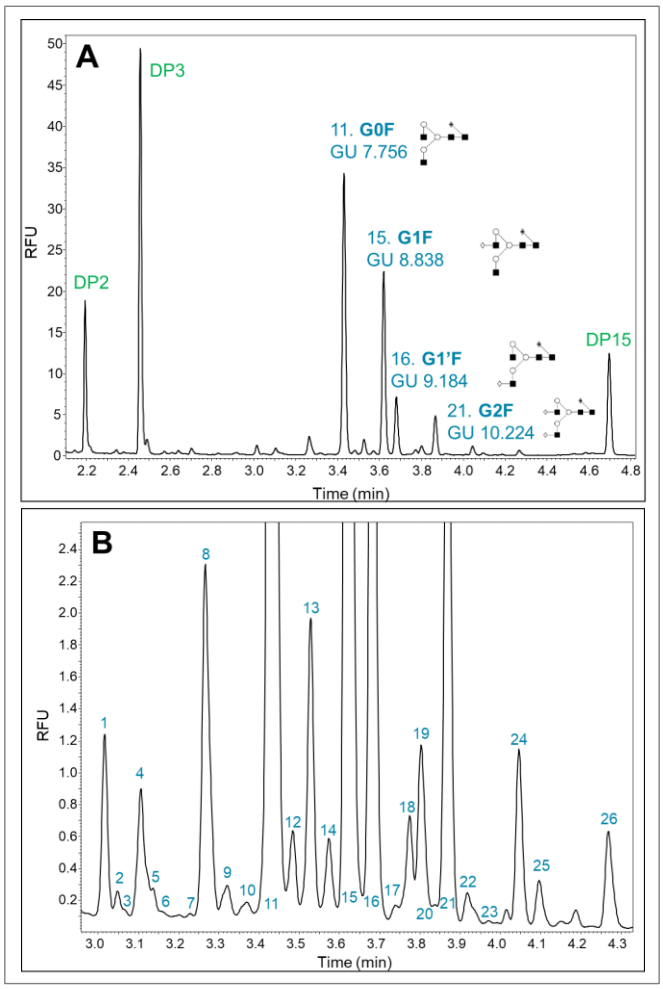

Figure 4. A. Fast CE-LIF glycan analysis of the NISTmAb. B. Zoomed view of the assay. Structures were verified by exoglycosidase sequencing.[5]

	*R.A. (%)	Oxford ID	Glycan ID
1	1.26	FA1G1S1	G1FS1-N
2	0.16	A2[6]G1S1	G1S1
3	0.03	FM3	Man3F
4	1.19	FA1 A2[3]G1S1	G0-N G1'S1
5	0.18	FA3G1S1	G3FS1
6	0.01	FA2[6]G1S1	G1FS1
7	0.01	FA2[3]G1S1	G1'FS1
8	3.30	A2 A1[6]G1	G0 G1
9	0.36	M5	Man5
10	0.21	FA1[6]G1 FA2G2S1	G1F-N G2FS1
11	41.49	FA2G2Ga1S1 FA2 M6	G2FS1+αGal G0F Man6
12	0.79	A2B	G0B
13	2.45	A2(3)G1	G1'
14	0.76	FA3	G0F+N
15	26.91	FA2(6)G1/ FM4A2G1 M7	G1F Man4FG1 Man7
16	8.94	FA2(3)G1	G1'F
17	0.04	FA2B[6]G1	G1FB
18	0.94	A2G2 FA1[6]G1Ga1	G2 G1F+αGal
19	1.64	M8/ FA1[3]G1Ga1 FA3G1	Man8/ G1'F-N+αGal G1F+N
20	0.12	FM5A1G1	Man5FGF-N
21	6.10	FA2G2	G2F
22	0.36	FA4 FA3G2	G0F+2N G2F+N
23	0.03	FA2BG2	G2FB
24	1.46	FA2G2[6]Ga1	G2F-αGal
25	0.41	FA2G2[3]Ga1	G2'F-αGal
26	0.85	FA2G2Ga2	G2F-(αGal)2

Table 1. Identities and relative abundances of the NISTmAb N-glycans. Underlined glycans are the most abundant of the co-migrating species.

Table 2 outlines the robustness and repeatability of the Fast Glycan Technology for selected key glycans (n=6). The assay maintains a high degree of robustness and repeatability while providing a rapid, simplified solution for glycan analysis.

Glycan	Relative Abundance (%)	Migration Time (%RSD)
G0F	41.49 ± 2.96	0.33
G1F	26.91 ± 2.17	0.31
G1'F	8.94 ± 2.51	0.32
G2F	6.10 ± 2.50	0.32

Table 2. Assay repeatability and robustness of the Fast Glycan Technology (n=6)

Rapid Charge Variant Profiling

CZE is a rapid and simplified approach to profile the charge heterogeneity of therapeutic mAbs. Simple sample preparation and inexpensive reagents make CZE an attractive approach for charge profile characterization. The rapid charge variant profile of the NISTmAb can be seen in Figure 5. The CZE assay resolved three distinct charge groups: the faster-migrating basic variants, the main group, and the slower-migrating acidic variants. NIST have previously identified the basic variants to be the heavy chain C-terminal lysine (K) variants.[6] The 2K and K variants migrate fastest and separate from the main peak. The later migrating and more complex acidic variants are known to include asparagine deamidation, lysine glycation, N-terminal glutamine and sialic acid glycovariants, however, specific peaks have not yet been identified.[7]

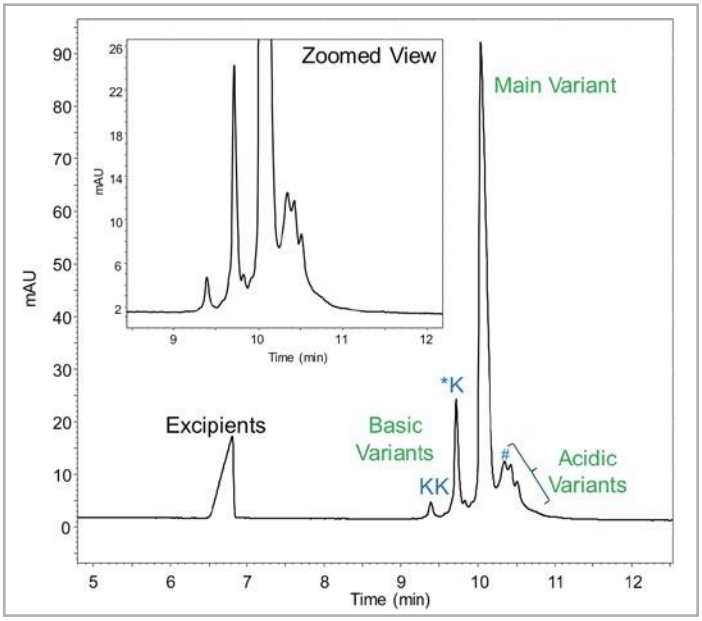

Figure 5. CZE charge profile of the NISTmAb.

The corrected areas (CA) of the basic, main, and acidic species were used to determine the relative abundance of each variant in the charge profiles according to equation 1:

$$Relative\ abundance_x\ (\%) = \frac{CA_x}{CA_{total}} \times 100 \qquad (1)$$

The CZE charge profiles are tabulated in Table 3. The results were very similar to those generated by NIST using a self- assembled cartridge with a 40 cm effective length.[6,7]

	Relative Abundance (%)	Migration Time (%RSD)
Basic	11.71 ± 0.09	*0.47
Main	72.99 ± 0.15	0.45
Acidic	15.29 ± 0.09	#0.44
Note * corresponds to the migration time of the basic variant and # corresponds to the migration time of the acidic variant.		

Table 3. Charge profile relative abundances of the NISTmAb variants and migration time repeatability (n=3).

Purity Determination

Traditionally, purity has been characterized by slab gel SDS PAGE, but this approach is labor intensive and results are only semi-quantitative with poor accuracy and precision. In contrast, the automated Purity assay of the PA 800 Plus in conjunction with the ready-to-use EZ-CE cartridge affords quantitative, highly accurate, and precise results.

Glycan occupancy was determined by analyzing the mAb in the reduced state (Figure 6A). Analysis of the non-reduced mAb was employed to assess monomeric purity (Figure 6B). The reduced assay was achieved in just 12 minutes, while the non-reduced assay required just 18 minutes (including aggregates peaks). The flat baseline of the Purity assay enabled the aggregate peaks to be visualized (Figure 6B insert).

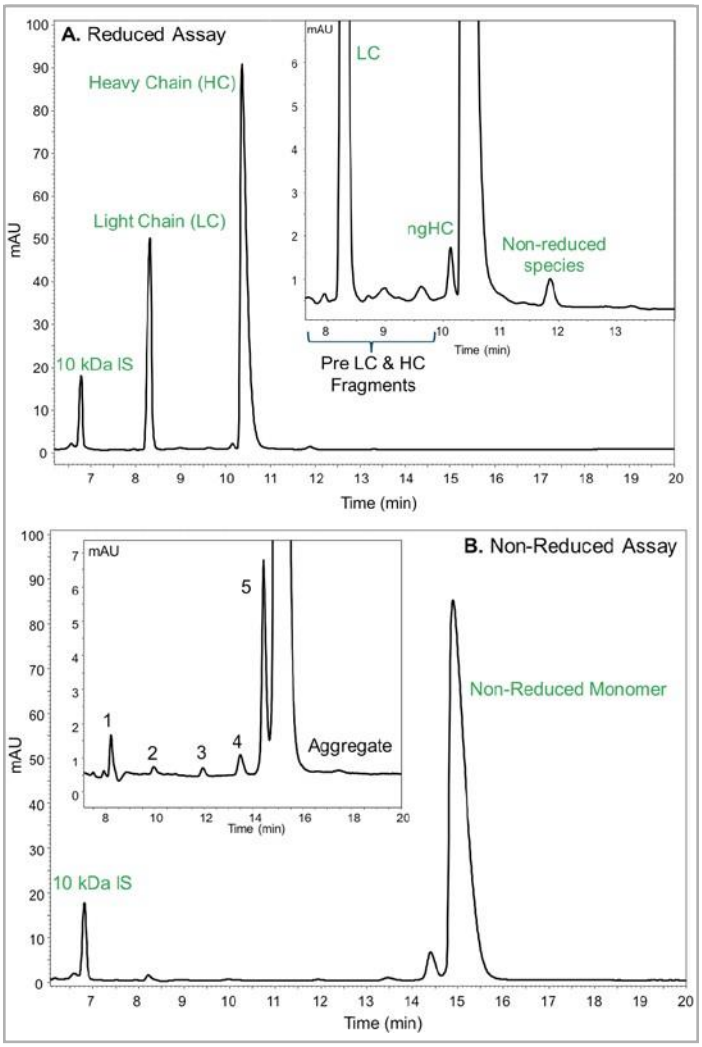

Figure 6. A. Reduced assay for glycan occupancy assess the abundance of non-glycosylated heavy chain (ngHC). B. Non-reduced assay for monomeric purity. Fragment impurities include: 1. Free light chain (LC), 2. Free heavy chain (HC), 3. HC:LC, 4. HC:HC, 5. HC:HC:LC.

The glycan occupancy and monomeric purity were calculated using equations 2 and 3. The results are listed in Table 4.

$$Glycan\ Occupancy\ (\%) = \frac{CA_{HC}}{CA_{HC} + ngCA_{HC}} \times 100 \qquad (2)$$

$$Monomeric\ Purity\ (\%) = \frac{CA_{monomer}}{CA_{monomer} + \sum CA_{fragments}} \times 100 \qquad (3)$$

	Reduced Assay	Non-Reduced Assay
Glycan Occupancy %	99.42 ± 0.02	
Non-Reduced Impurity %	0.37 ± 0.01	
Monomeric Purity %		94.45 ± 0.04
Migration Time %RSD	0.29	0.33
Peak Area %RSD	0.5	0.32

Table 4. Glycan occupancy and monomeric purity of the NISTmAb, and assay robustness.

Results obtained for both the glycan occupancy and monomeric purity were very similar to results described by NIST.[6,7] There are two likely explanations for any observed differences. The first, is that the flat base line of this Purity assay enables fragment peaks to be identified and integrated more effectively. The second, is possible differences in how the samples were stored.

The robustness and repeatability of the assays were measured by peak area and actual migration time percent RSD (n=6). Figures 7 and 8 display the overlaid assays and clearly demonstrates the repeatability of the Purity assay.

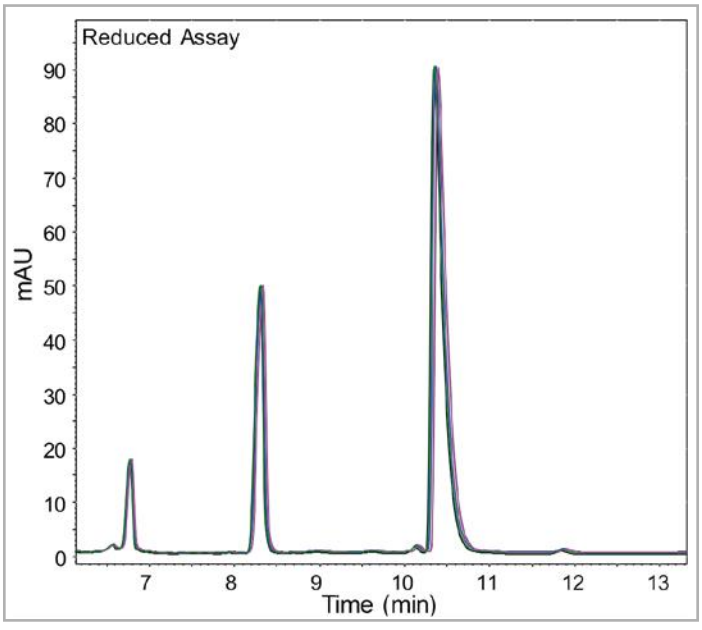

Figure 7. The Reduced Purity assay.

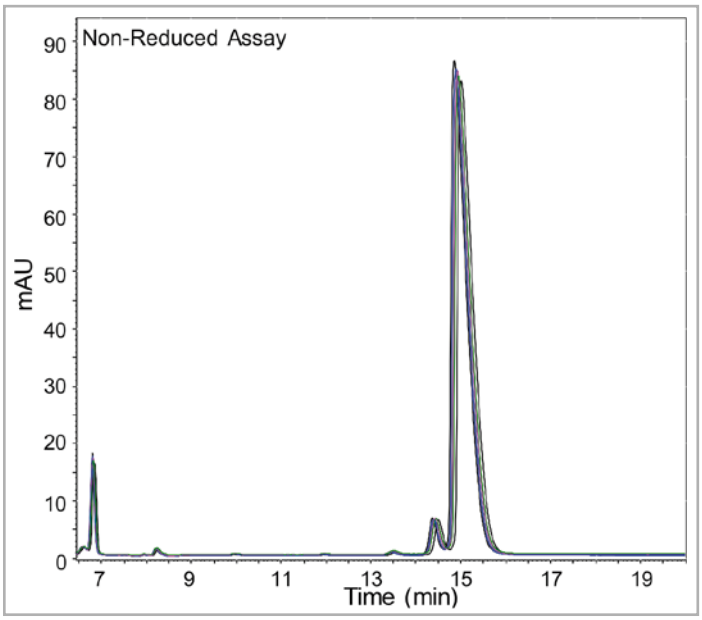

Figure 8. The Non-reduced Purity assay repeatability.

Conclusions

- The PA 800 Plus Pharmaceutical Analysis System was used to characterize the N-glycan microheterogeneity, charge heterogeneity and purity of the NISTmAb.
- Use of the EZ-CE cartridge and Fast Glycan Technology, allowed results to be achieved faster and with less effort.
- Results demonstrated excellent resolution and reproducibility, and were very similar to the results obtained by NIST, confirming the suitability of the PA 800 Plus Pharmaceutical Analysis System, EZ-CE cartridge, and Fast Glycan Technology for characterization of mAbs and mAb-based biotherapeutics.

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