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

単一分析プラットフォームにおける、NIST標準モノクローナル抗体の糖鎖解析・電荷不均一性評価・純度試験

Key Words：IgG抗体純度試験、電荷不均一性評価、糖鎖解析
PA800、IgGアッセイキット、ファーストグリカンラベリング解析キット、キャピラリーゾーン泳動法（CZE）

開発研究段階から、加速試験／安定性試験を含む品質管理試験まで、IgG抗体医薬品は様々な側面からの試験を必要としますが、これらを同一の分析技術により同一装置上で行うのは試験効率面からも大きなメリットがあります。
製薬支援キャピラリー電気泳動システムPA800 Plusは、この装置専用のキットを含む複数の解析手法、すなわち迅速な糖鎖解析キット／電化不均一性試験のためのキャピラリーゾーン泳動（CZE）法／純度試験に応用できるサイズ分離キットにより、多角的に解析します。それぞれの手法は近年さらに改良・改善を加えられ、単一ロットならすべてを半日で終えることも現実的です。
NIST標準モノクローナル抗体をサンプルとして、その実例を示しました。

糖鎖解析は、サンプル調製は磁気ビーズを用いて約1時間（Fig. 3）、泳動は10分以内（Fig. 4）の新しい方法がファーストグリカンラベリング解析キットにまとめられ、検出された糖鎖ピークの同定も自動化できます（Table 1）。
電荷不均一性試験目的に設定されたCZE法は、迅速であるとともに等電点電気泳動に匹敵する良好な分離（Fig. 5）と安定した再現性（Table 3）を提供します。
SDSキャピラリーゲル泳動は純度試験手法の標準として確立されていますが、この手法も泳動条件の見直しにより十分な分離を保ったままさらに迅速できます（Fig. 6）。頑堅性・再現性も十分確保されています（Fig. 7、8）。

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Fast Methods to Maximize the Use of the PA 800 Plus

Esme Candish,[1] Mervin Gutierrez,[1] Márton Szigeti,[2] Andras Guttman[1]
1 SCIEX Separations, Brea, CA;
2 Horváth Csaba Laboratory of Bioseparation Sciences, University of Debrecen, Hungary

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).

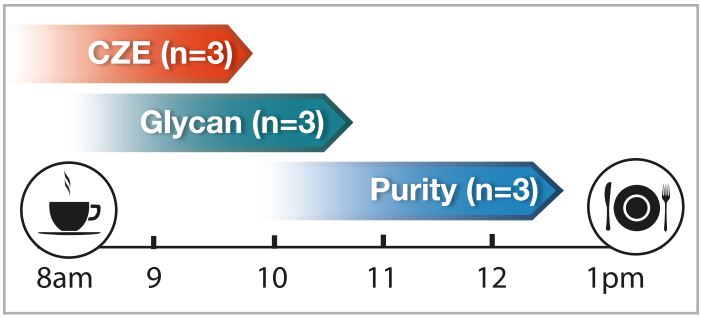

Figure 1. Flexible workflows on the PA 800 Plus help you accomplish a lot before noon.

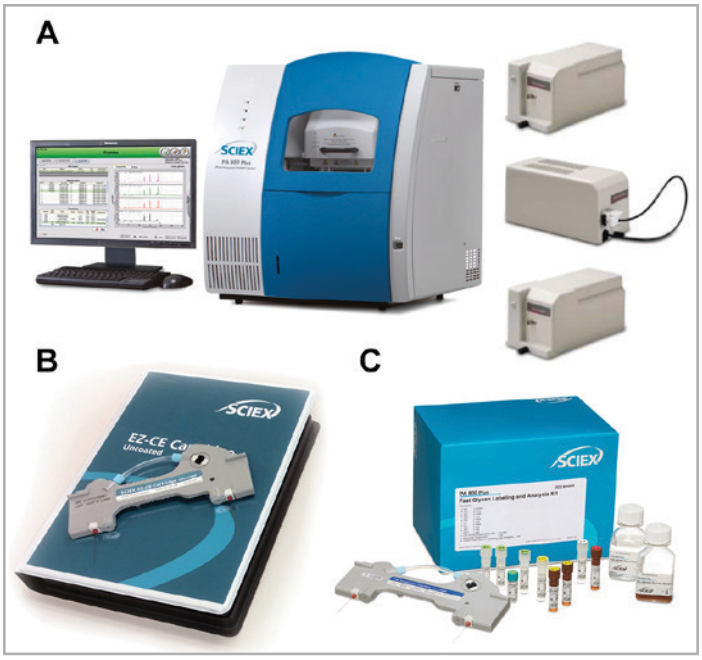

Figure 2. A. PA 800 Plus Pharmaceutical Analysis System
B. EZ-CE Cartridge C. Fast Glycan Technology

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]

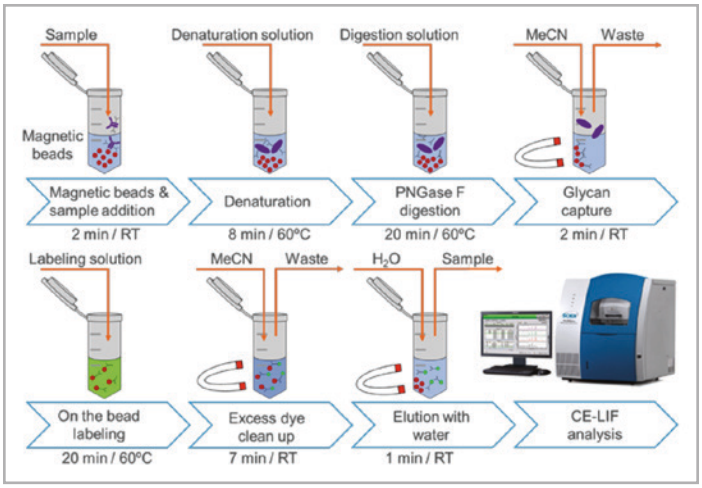

Figure 3. Workflow for the Fast Glycan Technology.

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]

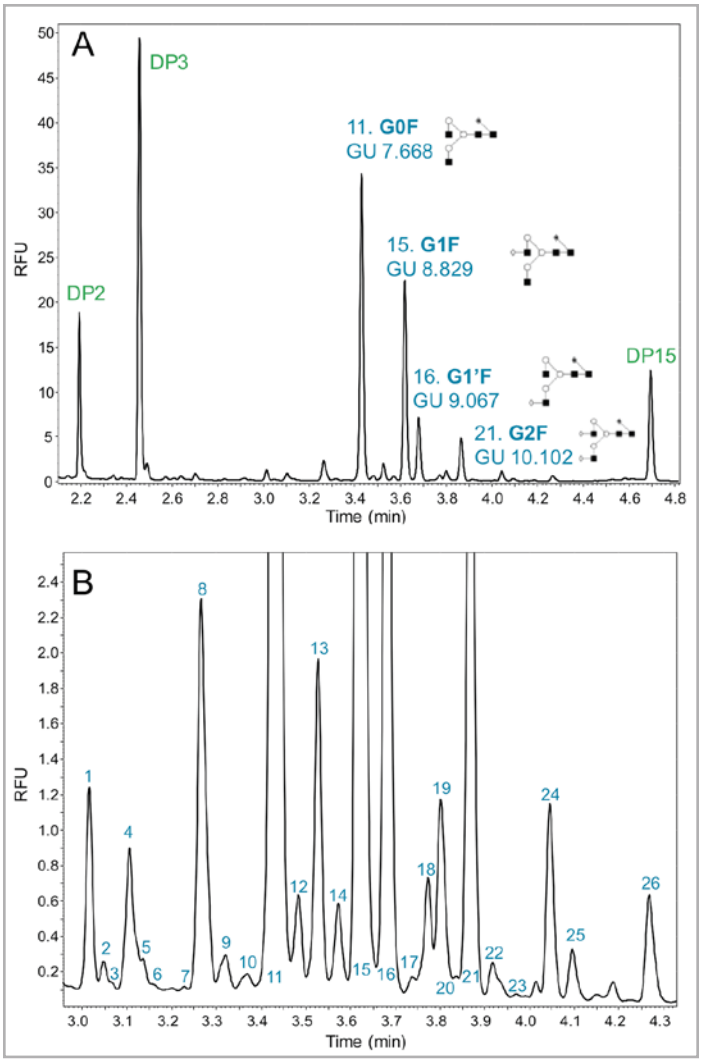

Figure 4. A. Fast CE-LIF glycan analysis of the NISTmAb. B. Zoomed view of the assay.

	GU	Oxford ID	Glycan ID	*R.A. (%)
1	5.385	FA1G1S1	G1FS1-N	1.26
2	5.530	A2[6]G1S1	G1S1	0.16
3	5.610	FM3	Man3F	0.03
4	5.852 5.917	FA1 A2[3]G1S1	G0-N G1'S1	1.19
5	5.992	FA3G1S1	G3FS1	0.18
6	6.109	FA2[6]G1S1	G1FS1	0.01
7	6.474	FA2[3]G1S1	G1'FS1	0.01
8	6.742 6.802	A2 A1[6]G1	G0 G1	3.30
9	6.987	M5	Man5	0.36
10	7.245 7.361	FA1[6]G1 FA2G2S1	G1F-N G2FS1	0.21
11	7.455 7.668 7.779	FA2G2Ga1S1 FA2 M6	G2FS1+αGal G0F Man6	41.29
12	7.918	A2B	G0B	0.79
13	8.200	A2(3)G1	G1'	2.45
14	8.467	FA3	G0F+N	0.76
15	8.729 8.865	FA2(6)G1/ FM4A2G1 M7	G1F Man4FG1 Man7	26.91
16	9.067	FA2(3)G1	G1'F	8.9
17	9.349	FA2B[6]G1	G1FB	0.04
18	9.946 9.590	A2G2 FA1[6]G1Ga1	G2 G1F+αGal	0.94
19	9.739 9.796	M8/ FA1[3]G1Ga1 FA3G1	Man8/ G1'F-N+αGal G1F+N	1.64
20	9.892	FM5A1G1	Man5FGF-N	0.12
21	10.102	FA2G2	G2F	6.10
22	10.393 10.681	FA4 FA3G2	G0F+2N G2F+N	0.36
23	10.801	FA2BG2	G2FB	0.03
24	11.101	FA2G2[6]Ga1	G2F-αGal	1.46
25	11.394	FA2G2[3]Ga1	G2'F-αGal	0.41
26	12.379	FA2G2Ga2	G2F-(αGal)2	0.85

Table 1. GU values, glycan identities and relative abundances of glycans of the NISTmAb. The underlined glycan species is the most abundant glycan 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	41.29 ± 2.96	0.33
G1F	26.91 ± 2.17	0.31
G1'F	8.90 ± 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]

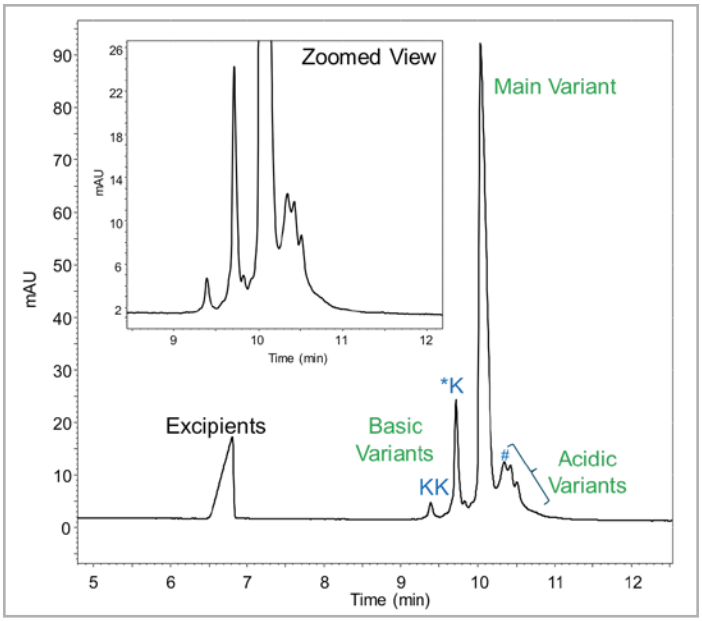

Figure 5. CZE charge profile of the NISTmAb.

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:

$$Relative\ Abundance_{\ x}\ (\%) = \frac{CA_x}{CA_{total}} \times 100 \quad (1)$$

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).

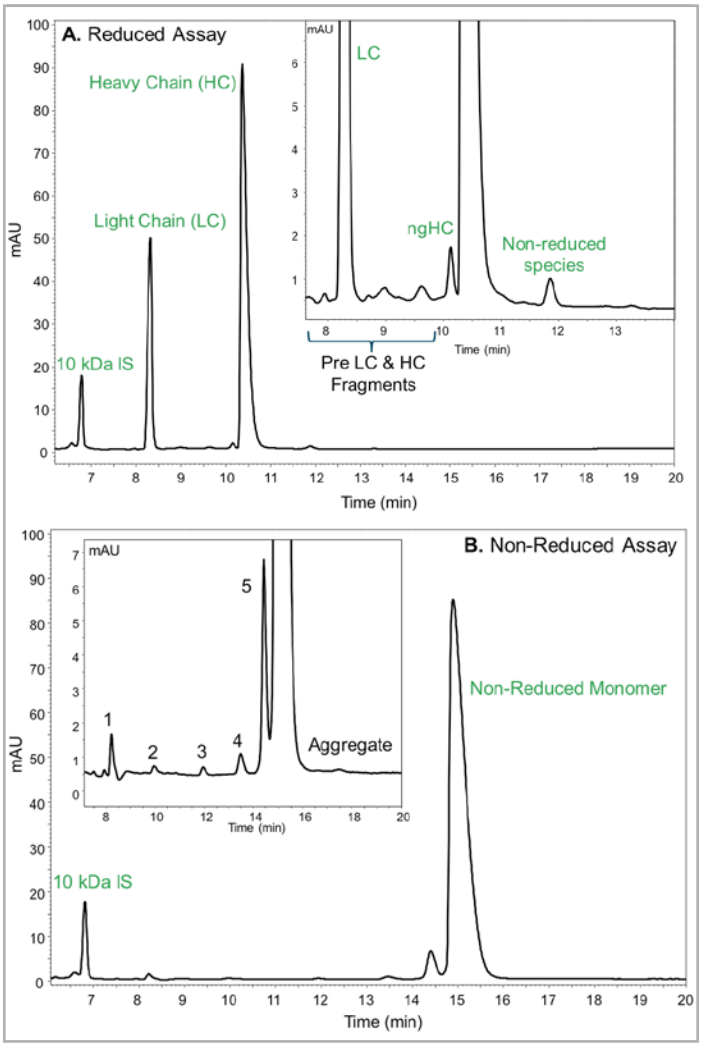

Figure 6. A. Reduced assay for glycan occupancy assess the abundance of non-glycosylated heavy chain (ngHC). B. Non-reduced assay for monomeric purity. Fragment impurities include: 1. Free light chain (LC), 2. Free heavy chain (HC), 3. HC:LC, 4. HC:HC, 5. HC:HC:LC.

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

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

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

	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). Figure 7 and 8 display the overlaid assays and clearly demonstrates the repeatability of the Purity assay.

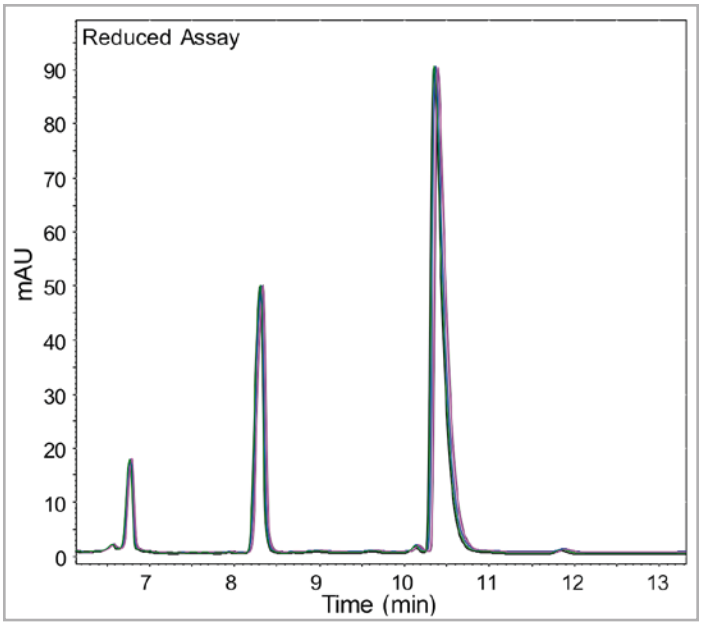

Figure 7. The Reduced Purity assay.

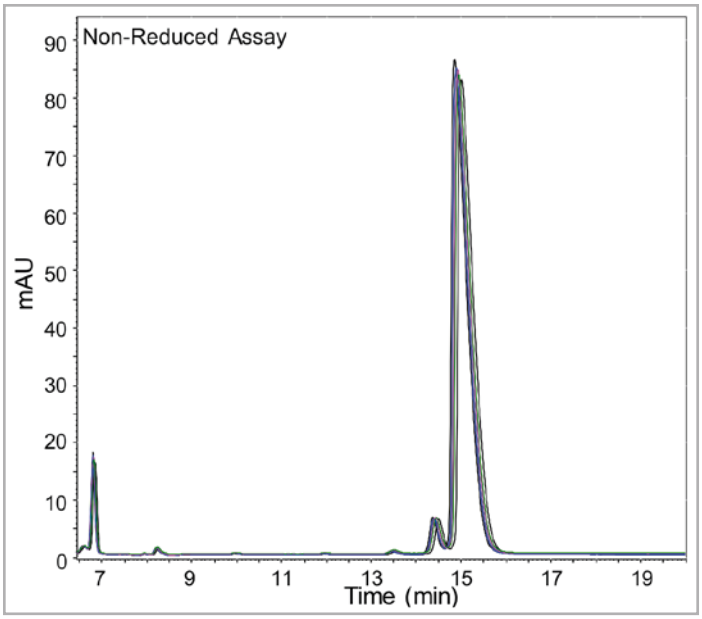

Figure 8. The Non-reduced Purity assay repeatability.

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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Headquarters
500 Old Connecticut Path, Framingham, MA 01701, USA
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