Technical Note

USP Monoclonal Antibody Reference Standards

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Monoclonal antibodies (mAb) continue to play an ever-increasing role in the pharmaceutical market. Half of the top-ten best-selling drugs in 2020 were mAbs which are also expected to dominate the future biosimilar market[1,2].

The development of these products requires comprehensive physicochemical and biophysical characterization before their approval. These analytical requirements can be challenging due to the complex nature of biologics, such as the susceptibility of these molecules to post-translational modifications that result in product heterogeneity. A well characterized reference standard that is widely available is a valuable tool for ensuring that tests are comparable between laboratories and deliver reliable and reproducible results.

In addition to USP's first mAb Reference Standard (RS), USP Monoclonal IgG System Suitability RS, which is referenced in *USP General Chapter <129> Analytical Procedures for Recombinant Therapeutic Monoclonal Antibodies*[3], USP has also developed three non-compendial monoclonal antibody RSs (mAb 001, mAb 002, and mAb 003) to provide a range of reference materials with different physicochemical properties (Table 1). This will allow users to select the most suitable RS for their purposes.

The mAb RSs can be used in a broad range of applications, serving as:

- Internal assay control
- Independent control material for method development
- Standardization of physicochemical testing, such as intact mass, charge heterogeneity, size variants, purity and glycan analyses across laboratories
- Development of platform technologies
- Proficiency controls for method transfer and staff training

General information on the USP mAb Reference Standards

All USP mAb RSs to date are recombinant humanized IgG1s that were expressed in Chinese hamster ovary (CHO) cell culture (currently the most common cell line used for mAb manufacturing) and were manufactured using industry standard upstream production and downstream purification.

The USP mAb RS have been rigorously tested and evaluated during multi-laboratory studies using the methods described in *USP General Chapters <129> Analytical Procedures for Recombinant Therapeutic Monoclonal Antibodies*[3], *<210> Monosaccharide Analysis*[4], *and <212> Oligosaccharide Analysis*[5], as well as in-house methods developed by participating collaborators.

Table 1. General information for the three non-compendial USP mAb Reference Standards

	USP mAb 001, monoclonal IgG1	USP mAb 002, monoclonal IgG1	USP mAb 003, monoclonal IgG1
USP Catalog #	1445539	1445547	1445595
CAS #	174722-31-7	216974-75-3	912628-39-8
MW	~147,000 Da	~150,000 Da	~146,000 Da
Theoretical pI*	8.7	8.1	8.1
Experimental pI (cIEF)**	9.2	7.8	7.7
Experimental pI (icIEF)**	9.2	7.9	7.8
Package size	200 µl solution (2 mg protein content)	200 µl solution (2 mg protein content)	200 µl solution (2 mg protein content)

** Calculated using ProtParam (ExPASy) without glycosylation*
***Per USP In-house methods, see charge variant application note for more details.*

Here we summarize the findings from some of the quality control assays which are routinely used to establish identity and purity of the product. Post-translational modifications such as glycosylation, N-terminal pyroglutamate and C-terminal lysine deletion were also studied as part of the RSs evaluation. Deamidation and oxidation were not included since they can vary widely depending on sample preparation and storage. The extensive characterization of the three mAb RS provides users the option to select the mAb that best reflects the key attributes they want to assess, saving time and costs associated with in-house evaluation of the RSs.

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The isoelectic point (pI) values of the three mAb were determined by Capillary Isoelectric Focusing (cIEF Need and imaged Capillary Isoelectric Focusing (icIEF)). More analytical details and results can be found in the USP Technical Application Note for mAb charge variant analysis but overall, mAb 002 and mAb 003 have a lower pI relative to mAb 001 and each mAb shows a unique profile of acidic and basic variants **(Table 1)**.

Capillary Electrophoresis Sodium Dodecyl Sulphate (CE- SDS) under reducing conditions was conducted according to the analytical conditions detailed in General Chapter <129>. The results **(Figure 2** and **Table 3)** indicate that the level of non-glycosylated heavy chain (NGHC) is lowest in mAb 001 (almost half of mAb002), thus mAb RS 001 is especially useful for establishing or testing method sensitivity.

CE-SDS analysis under non-reducing conditions was also carried out according to General Chapter <129>. Results shown in **Figure 3** and **Table 4**, demonstrate that mAb 003 contains a range of fragments, making it particularly useful for method development and qualification.

Size exclusion chromatography (SEC-HPLC) was used to determine the purity of the main peak, as well as high and low molecular weight impurities of the RS following the analytical method described in General Chapter <129>. Results (see **Figure 1 and Table 2**) indicate that mAb 001 has the most complex population of dimer and higher order aggregates, noted as high molecular weight species (HMWS), whereas mAb 002 and mAb 003 show better resolution between the monomer and aggregate.

Glycoprofiling is a key assay for lot release and characterization of manufactured glycoproteins, both before and after approval. The glycan heterogeneity of the mAb RSs were studied at both the intact and released glycan level.

The theoretical masses of mAb 001, mAb 002, and mAb 003 were confirmed by intact mass analyses, which was performed using in-house LC-High Resolution Mass Spectrometry (HRMS) methods (deglycosylated intact protein, reduced protein, and reduced deglycosylated protein; data not shown). The study also showed differences in the relative abundance of glycoforms. For example, for mAb 002, the G0F/G0F peak yielded the highest response, while for mAb 001 and mAb 003 the highest response was observed for G0F/G1F; see **Figure 4** and **Table 5**.

Glycoprofiling was also expanded to evaluate the released N-glycans in more detail by a variety of methods, such as Capillary Electrophoresis-Laser-Induced Fluorescence (CE-LIF, General Chapter <212>) and Hydrophilic Interaction Chromatography with Fluorescence and Mass Spectrometry (HILIC-FLR-MS, with 2-aminobenzamide - 2-AB and RapiFluor-MS™ labeling). N-glycan structures and relative intensities of each glycan of mAb 001, mAb 002 and mAb 003 are described in **Figures 5 - 6** and **Tables 6 - 7**. The differences in the proportion of terminal galactose of the major species provides a variety of glycan profiles.

Both intact mass and peptide mapping studies showed post-translational modifications, including N-terminal pyroglutamate in both the light and heavy chain of mAb 001, and C-terminal lysine truncation in the heavy chain of all three antibodies **(Table 8)**.

Sialic acid analysis was performed using both High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) and LC-FLR method according to General Chapter <210>. Results are shown in **Table 9**.

The sialic acid analysis result expressed as molar ratios shows that levels of *N*-acetylneuraminic acid (Neu5Ac) are very low in all 3 mAb RSs, and *N*-Glycolylneuraminic acid (Neu5Gc) is not detected.

Conclusions

The USP mAb Reference Standards provide users with options to select the best fit-for-purpose RS for their specific analytical needs. The physicochemical diversity shown by the three mAb RSs makes them highly versatile and suitable for a broad range of applications. USP and collaborators continue to evaluate these materials for additional uses and will publish additional technical application notes in the future. These mAb RSs are another example of USP's continuous commitment to support access to high quality medicines.

At the time of this release, USP is evaluating the use of RSs in additional applications, including Multi-Attribute Method (MAM). To learn more about USP's MAM knowledge hub, an online community of scientists and experts dedicated to accelerating MAM scientific knowledge, register at mam.usp.org.

References

1. IQVIA (MIDAS) quarterly data
2. Frost & Sullivan Access to new therapy areas to drive major growth in the global biosimilars market 2020-2026
3. *USP-NF General Chapter <129> Analytical Procedures for Recombinant Therapeutic Monoclonal Antibodies*
4. *USP-NF General Chapter <210> Monosaccharide Analysis*
5. *USP-NF General Chapter <212> Oligosaccharide Analysis*

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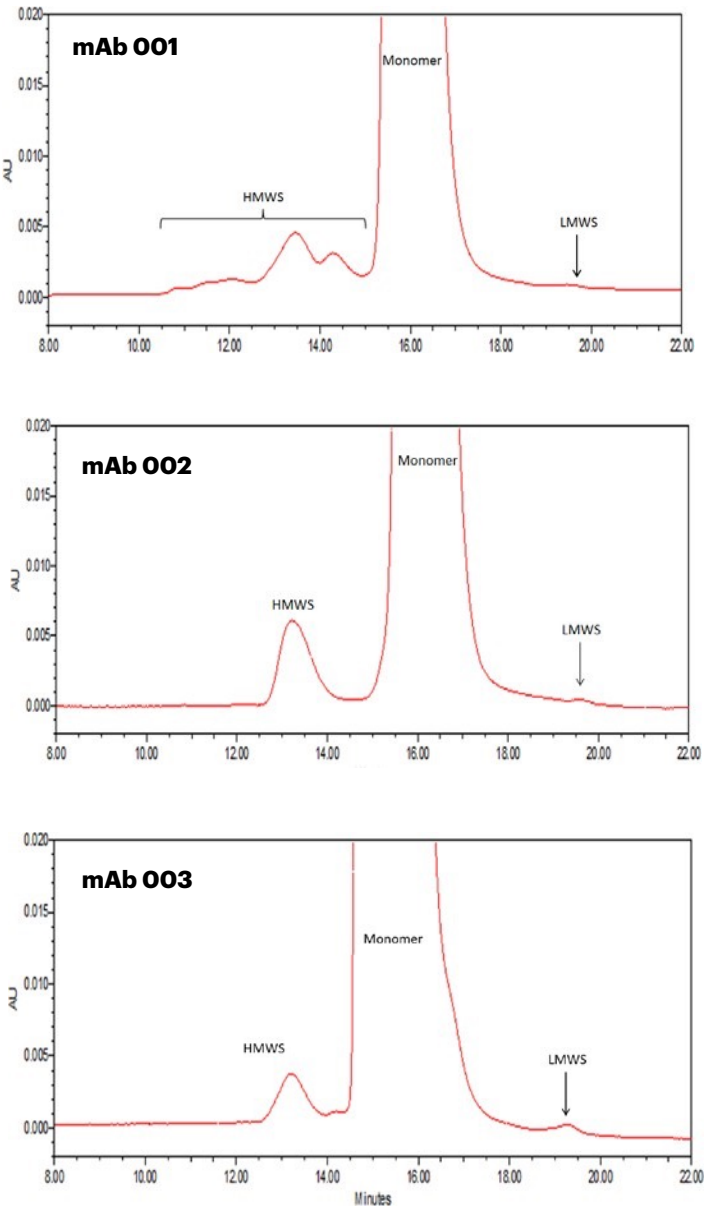
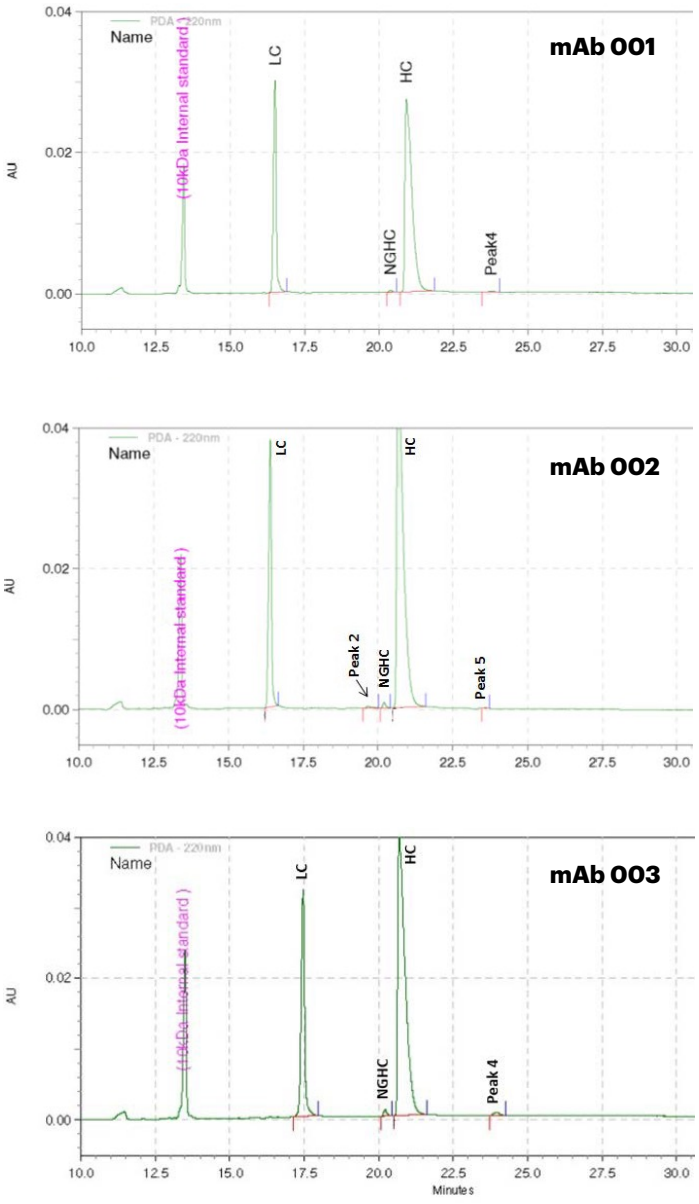


Figure 1. RS analysis by Size Exclusion Chromatography (SEC-HPLC). Method: General Chapter <129>
(https://doi.usp.org/USPNF/USPNF_M6297_02_01.html)



Zoomed scale Electropherogram

Figure 2. RS analysis by Capillary SDS Electrophoresis (CE-SDS), reducing conditions.
Method: General Chapter <129>
(https://doi.usp.org/USPNF/USPNF_M6297_02_01.html)

Table 2. mAb species determined by SEC-HPLC (General Chapter <129>)

Reference Standard	Species (% TDA)		
	HMWS	Monomer	LMWS
mAb 001	0.9	99.1	< 0.1
mAb 002	0.8	99.2	< 0.1
mAb 003	0.4	99.6	< 0.1

TDA: Total Detected Area; HMWS: High Molecular Weight Species; LMWS: Low Molecular Weight Species

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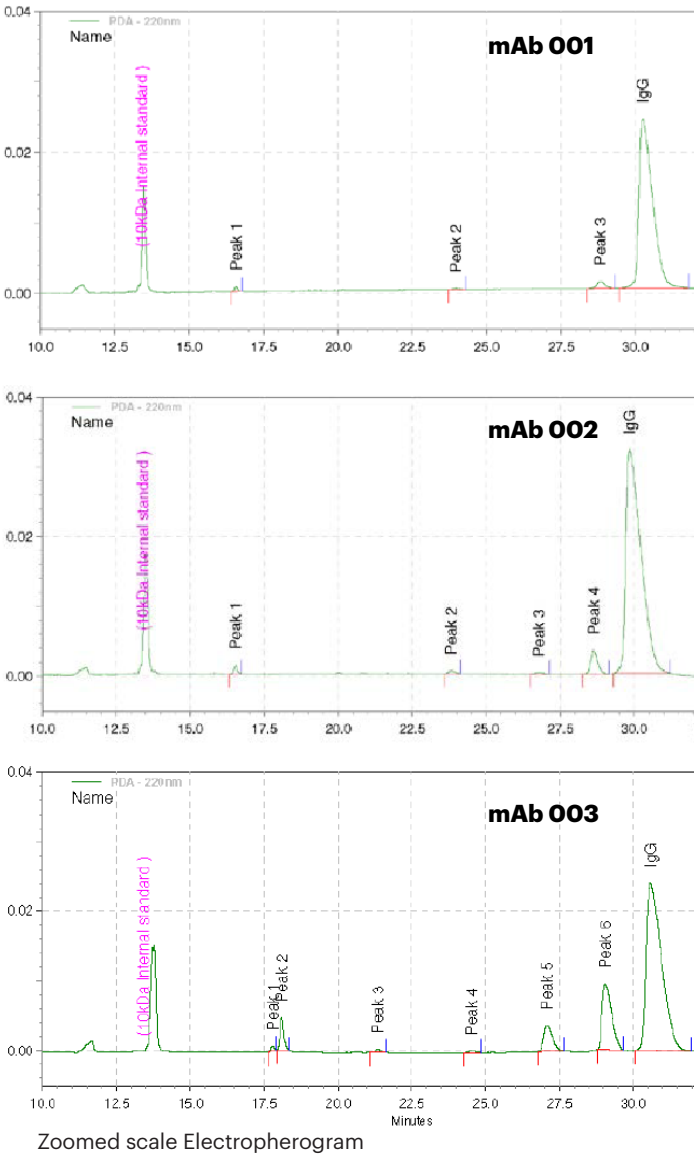

Zoomed scale Electropherogram

Figure 3. IgG content analysis by CE-SDS, non-reducing conditions. Method: General Chapter <129> (https://doi.usp.org/USPNF/USPNF_M6297_02_01.html)

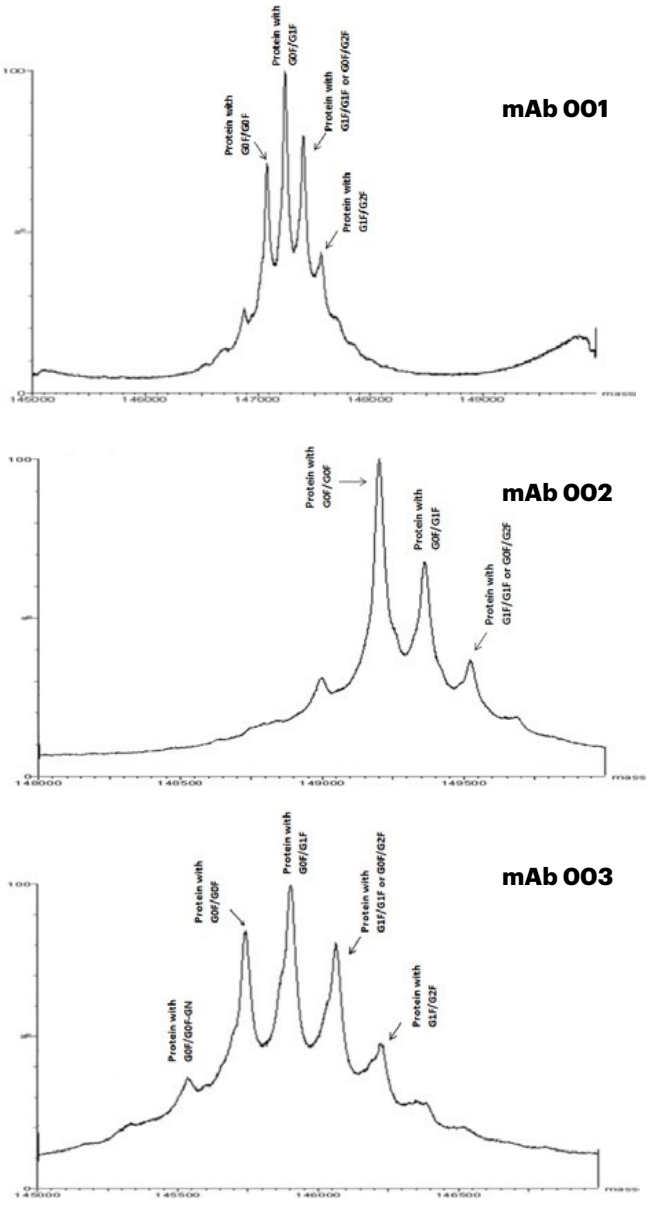

Figure 4. Intact Protein Mass Analysis of RS (deconvoluted mass spectrum)

Table 3. mAb species determined by CE-SDS, reducing conditions (General Chapter <129>)

Reference Standard	Species (% TDA)			
	LC	NGHC	HC	Total impurity peaks
mAb 001	34.1	0.3	65.4	0.3
mAb 002	31.4	0.6	67.5	0.5
mAb 003	29.8	0.8	68.8	0.7

TDA: Total Detected Area; LC: Light Chain;
NGHC: Non-Glycosylated Heavy Chain; HC: Heavy Chain

Table 4. IgG content analysis by CE-SDS, non-reducing conditions (General Chapter <129>)

Reference Standard	IgG main peak (%)
mAb 001	95
mAb 002	93
mAb 003	69

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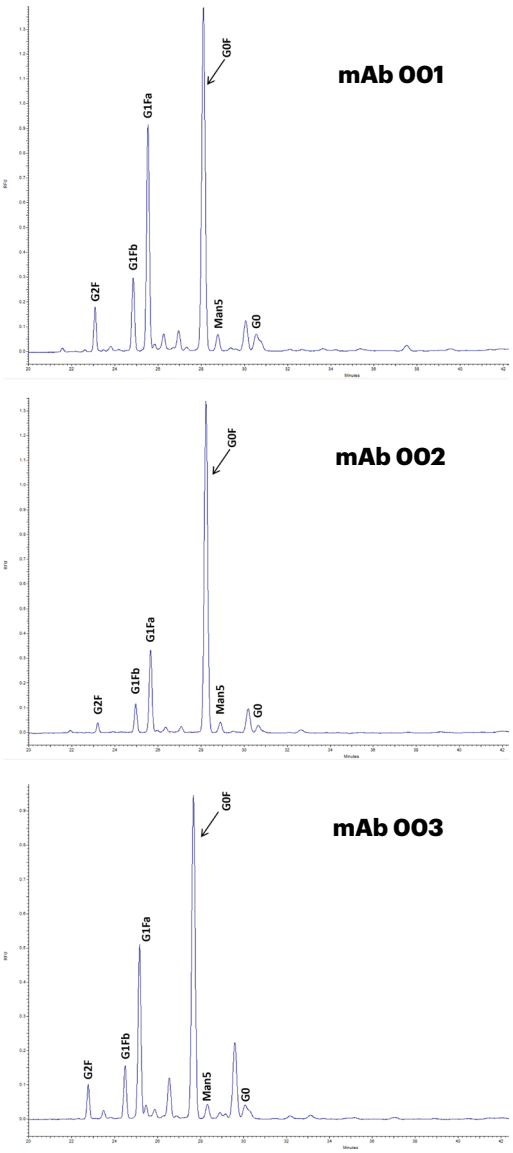

Zoomed scale Electropherogram

Figure 5. N-linked Oligosaccharide Analysis by CE-LIF. Method <212> (USP42/NF37)

Table 5. RS Composition by intact protein mass analysis.

Reference Standard	Composition (Theoretical Average MW)*			
	Intact protein with G0F/G0F	Intact protein with G0F/G1F	Intact protein with G1F/G1F or G0F/G2F	Intact protein with G1F/G2F
mAb 001	147,075.2	147,237.4	147,399.6	147,561.7
mAb 002	149,197.5	149,359.7	149,521.9	ND[1]
mAb 003	145,737.4	145,899.6	146,061.8	146,223.9

**All theoretical masses are calculated by Thermo BioPharma Finder™ software, with assumption of N-term pyro Glu (NH3 loss) (mAb 001) and C-term Lys truncation (mAb 001, mAb 002, mAb 003).*

[1]ND: Not Detected

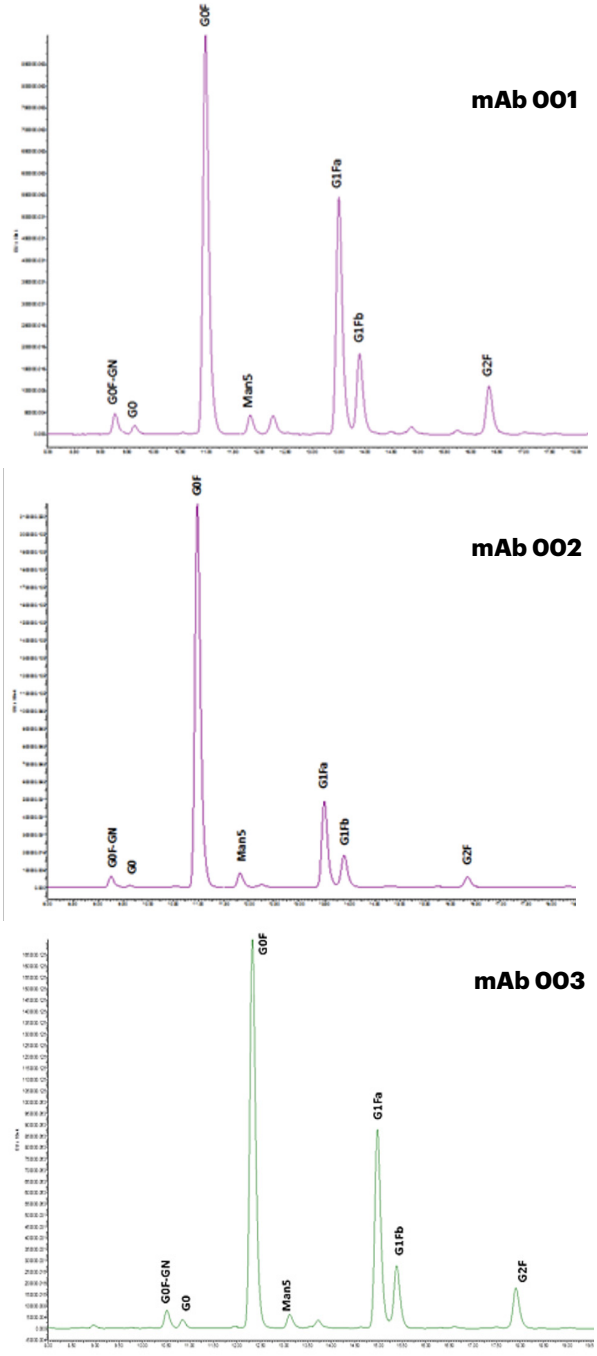

Figure 6. Analysis of N-Linked Oligosaccharide of RS by HILIC LC-FLR-MS (2-AB labeling)

Table 6. *N*-Glycan analysis by CE-LIF (General Chapter <212>)

Reference Standard	Relative TDA (%)					
	G2F	G1Fb	G1Fa	G0F	Man 5	G0
mAb 001	5.04	9.16	27.86	49.87	3.40	4.68
mAb 002	1.78	5.42	15.31	70.56	4.18	2.76
mAb 003	4.24	7.67	25.20	54.18	4.16	4.55

TDA: Total Detected Area

Table 7. *N*-Glycan analysis by HILIC LC-FLR-MS (General Chapter <212>)

Reference Standard	Relative TDA (%)					
	G0F-GN	G0	G0F	G1Fa	G1Fb	G2F
mAb 001	2.28	1.17	48.74	30.42	10.85	6.53
mAb 002	1.89	0.31	71.65	16.95	6.87	2.33
mAb 003	2.18	1.19	53.15	28.16	9.36	5.94

Table 8. Post-translational modification (PTM) analysis - Pyroglutamate and Lysine truncation

Reference Standard	PTM %		
	Light Chain	Heavy Chain	
	N-term Pyro Glu	N-term Pyro Glu	C-term Lys
mAb 001	95.9	98.7	95.7
mAb 002	-	1.1	98.6
mAb 003	-	1.5	90.7

PTM: Post-translational modification

Table 9. Molar ratio of sialic acid to protein (nmol / nmol) determined by two methods (General Chapter <210>)

Reference Standard	Neu5Ac / protein (nmol / nmol)	Neu5Gc / protein (nmol / nmol)
mAb 001	0.05	ND
mAb 002	0.02	ND
mAb 003	0.03	ND

ND: Not Detected

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