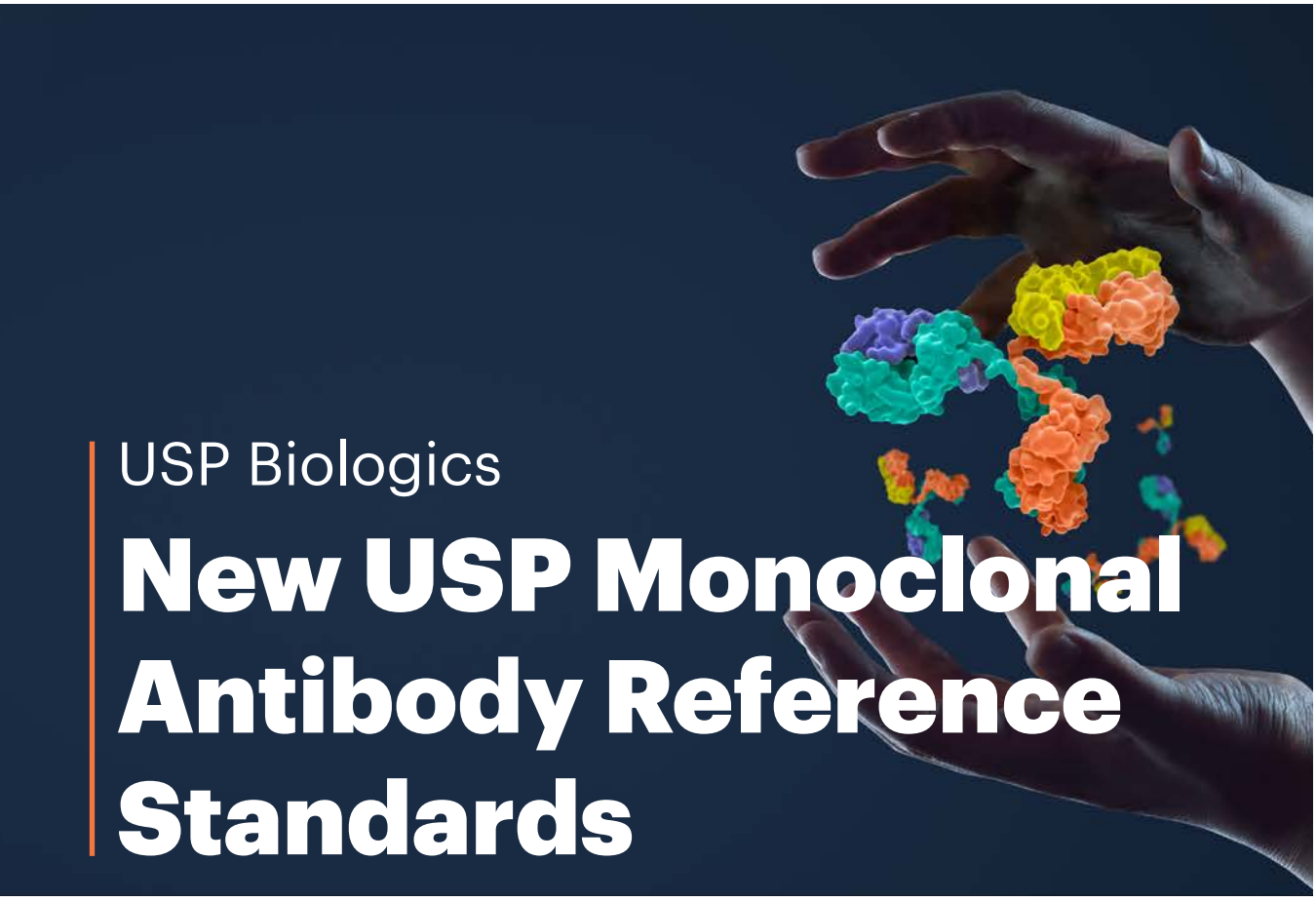

Technical Note

USP Biologics

New USP Monoclonal Antibody Reference Standards

Monoclonal antibodies (mAb) continue to play an ever-increasing role in the pharmaceutical market. More than half of the top-ten best-selling drugs in 2019 were mAbs[1,2]. Applications include treatments from cancer to autoimmune disorders and macular degeneration. The development of such products requires comprehensive physicochemical and biophysical characterization before their approval. A 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 the US, more than 80 mAbs have received approval by FDA. However, the majority do not have a reference standard. Moreover, due to the inherent heterogeneity and complexity of this type of molecule, existing mAb standards do not cover all the analytical needs.

USP has developed three new non-compendial monoclonal antibody Reference Standards (mAb 001, mAb 002, and mAb 003) to overcome the limited availability of mAb standards and to provide a range of reference materials with different physico-chemical properties (**Table 1**). This will allow users to select the most suitable Reference Standard (RS) for their purpose.

The intended use of the mAb RS is as a qualitative standard in a broad range of applications:

- 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.

In addition, the mAb RS can be used for activities such as training and method transfer.

General information on the mAb Reference Standards

All mAb RS are recombinant humanized IgG1 that were expressed in Chinese hamster ovary (CHO) cell culture and have undergone industry standard upstream and downstream purification.

The new USP Reference Standards have been rigorously tested and evaluated in inter-laboratory studies using methods described in USP 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 new 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
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)*

The evaluation of the three mAb RS determined that each contains two identical light chains and two identical heavy chains linked through inter- and intra-chain disulfide bonds. Their post-translational modifications (PTM) include deamidation, methionine oxidation, tryptophan oxidation, N-terminal pyroglutamate, C-terminal lysine deletion, and *N*-glycosylation. This document shows information on glycosylation, N-terminal pyroglutamate and C-terminal lysine deletion. Deamidation and oxidation are not included since they can vary widely depending on sample preparation and storage. The extensive evaluation of the three mAb RS allowed the comparison of their physico-chemical characteristics. This is relevant as it allowed the identification of specific attributes that can help the user in the selection of the most suitable RS for their specific needs, saving time and costs associated with in-house evaluation of the Reference Standards.

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 Chapter <129>. Results (**Figure 1** and **Table 2**) indicate that mAb 001 has the most complex high molecular weight species (HMWS), whereas mAb 002 and mAb 003 show better resolution between the monomer and aggregate. This provides the user an option to select the mAb that best reflects the key attributes they want to assess during method development and qualification.

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

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

Intact mass analyses were 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 confirms theoretical mass of mAb 001, mAb002, and mAb 003, and also shows that 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 (**Figure 4** and **Table 5**).

Glycoprofiling is a key assay for the lot release characterization of manufactured glycoproteins both before and after approval. In this study, glycoprofiling was expanded to evaluate the release *N*-glycan in more detail by a variety of methods, such as Capillary Electrophoresis-Laser-Induced Fluorescence (CE-LIF, Chapter <212>), and Hydrophilic Interaction Chromatography with fluorescence and Mass Spectrometry (HILIC-FLR-MS, with 2-aminobenzamide - 2-AB - and *Rapi*Fluor-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 proportion of terminal galactose of the major species provide 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 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 mAbs, and *N*-Glycolylneuraminic acid (Neu5Gc) are not detected.

The isoelectic point (pI) values of the three mAb were determined by Capillary Isoelectric Focusing (cIEF) using a Sciex PA 800 Plus System. As shown in **Tables 1** & **10** and **Figure 7**, 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. The variation in pI and charge variant profiles between mAbs allows the user to select the mAb that most closely reflects their specific attributes of interest.

Conclusions

The new USP mAb Reference Standards contribute to the physico-chemical diversity of available RS, which aims to help users find the best fit-for-purpose RS for their needs.

The physico-chemical diversity shown by the three mAb RS makes them highly versatile and suitable for a broad number of uses and applications. At the time of release of the three mAb RS, USP is evaluating the use of the RS in additional applications, including icIEF, peptide mapping or Multiattribute Method (MAM).

The three new mAb Reference Standards are another example of USP's continuous commitment to provide the foundation for high-quality medicines.

References

1. https://biopharmadealmakers.nature.com/users/9880-biopharma-dealmakers/posts/53687-moving-up-with-the-monoclonals
2. https://www.pharmaceutical-technology.com/features/top-selling-prescription-drugs/
3. Chapter <129> Analytical Procedures for Recombinant Therapeutic Monoclonal Antibodies (USP42/NF37)
4. Chapter <210> Monosaccharide Analysis (USP42/NF37)
5. Chapter <212> Oligosaccharide Analysis (USP42/NF37)

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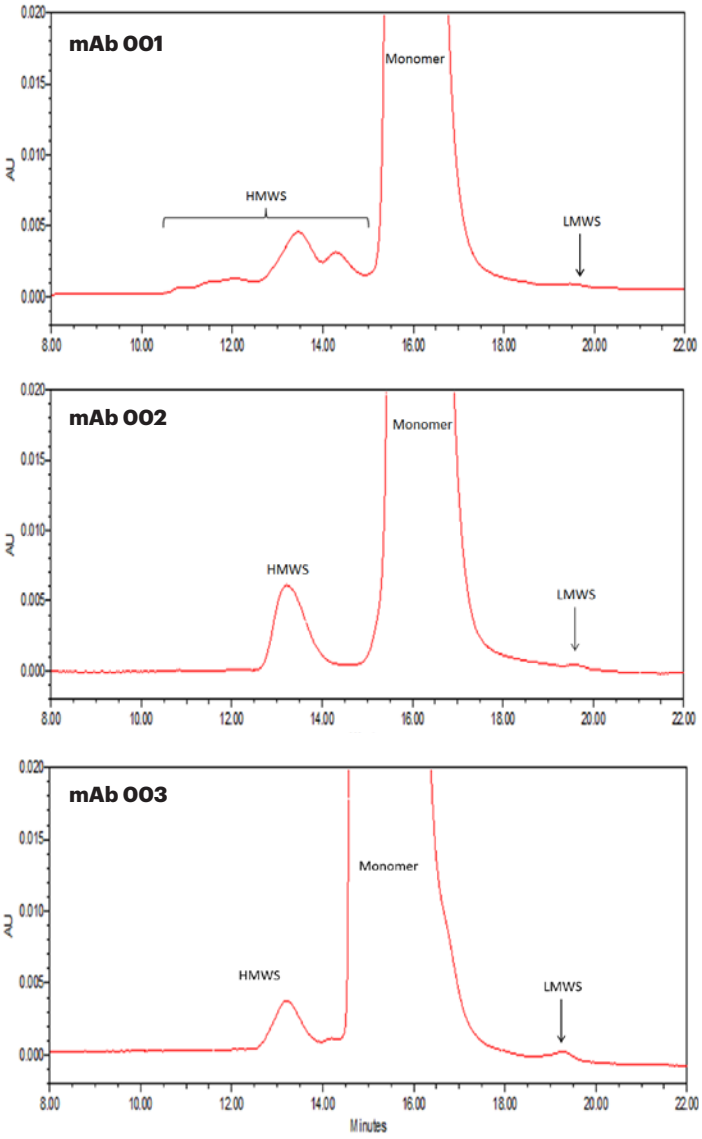

Figure 1. RS analysis by Size Exclusion Chromatography (SEC-HPLC). Method: Chapter <129> (USP42/NF37)

Table 2. mAb species determined by SEC-HPLC (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

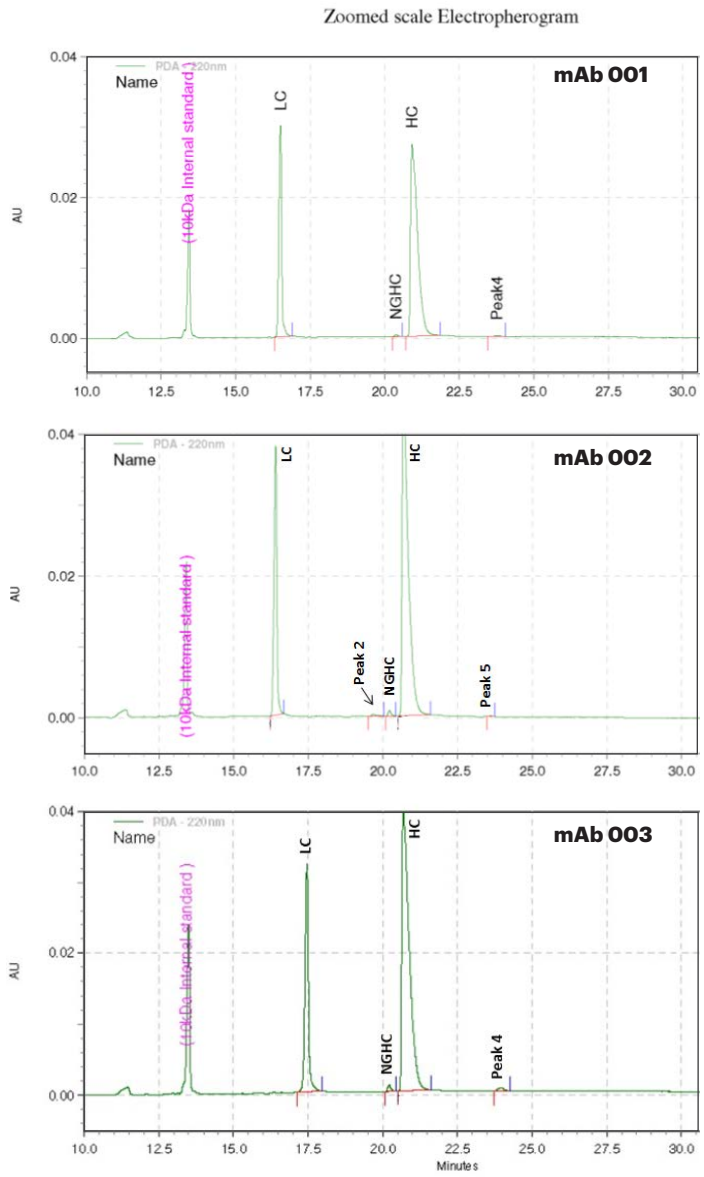

Figure 2. RS analysis by Capillary SDS Electrophoresis (CE-SDS), reducing conditions. Method: Chapter <129> (USP42/NF37)

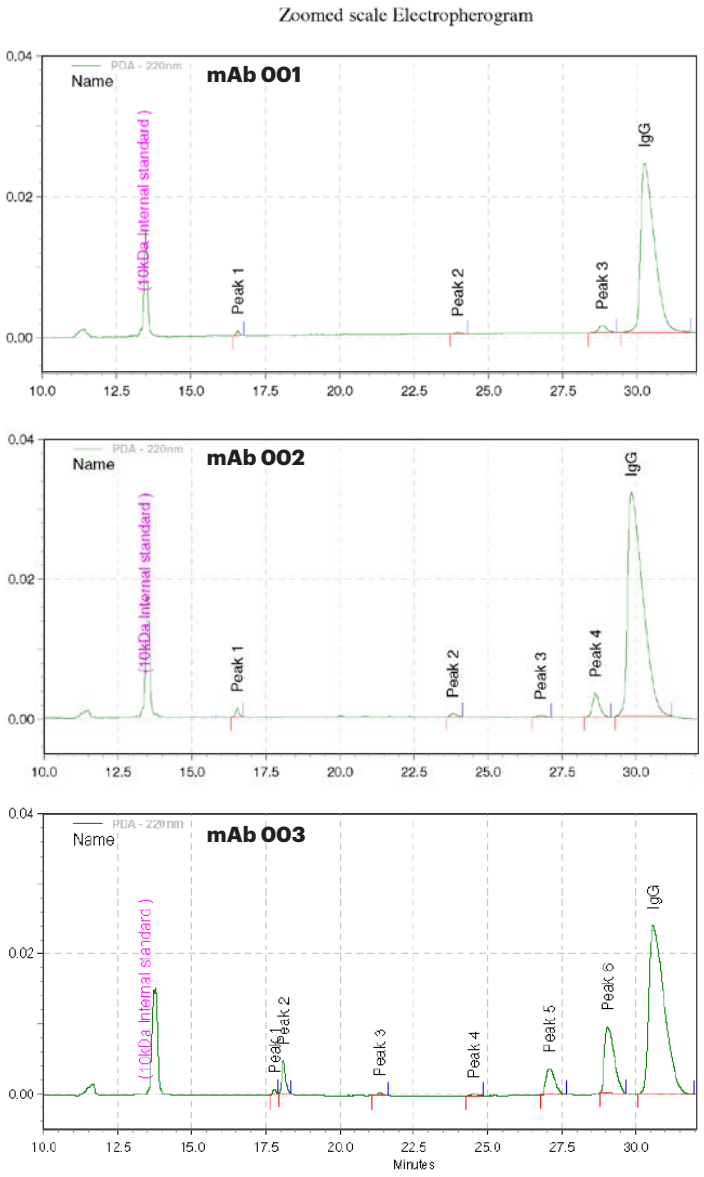

Figure 3. IgG content analyzed by CE-SDS, non-reducing conditions. Method: Chapter <129> (USP42/NF37)

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

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

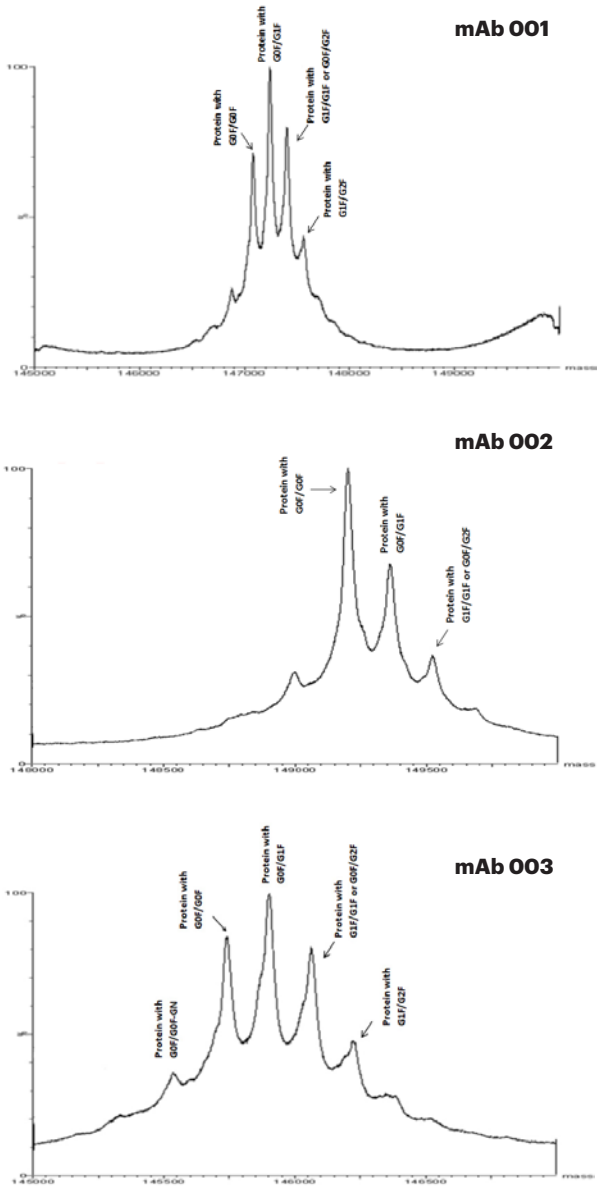

Figure 4. Intact Protein Mass Analysis of RS (Deconvoluted mass spectrum)

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

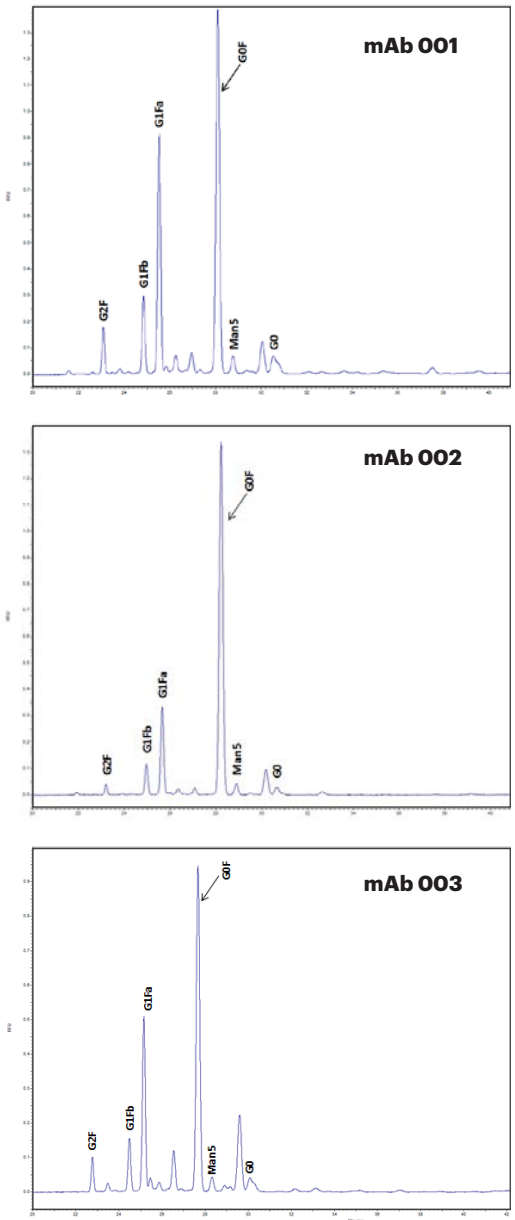

Figure 5. *N*-linked Oligosaccharide Analysis by CE-LIF. Method <212> (USP42/NF37)

Table 6. *N*-Glycan analysis by CE-LIF (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

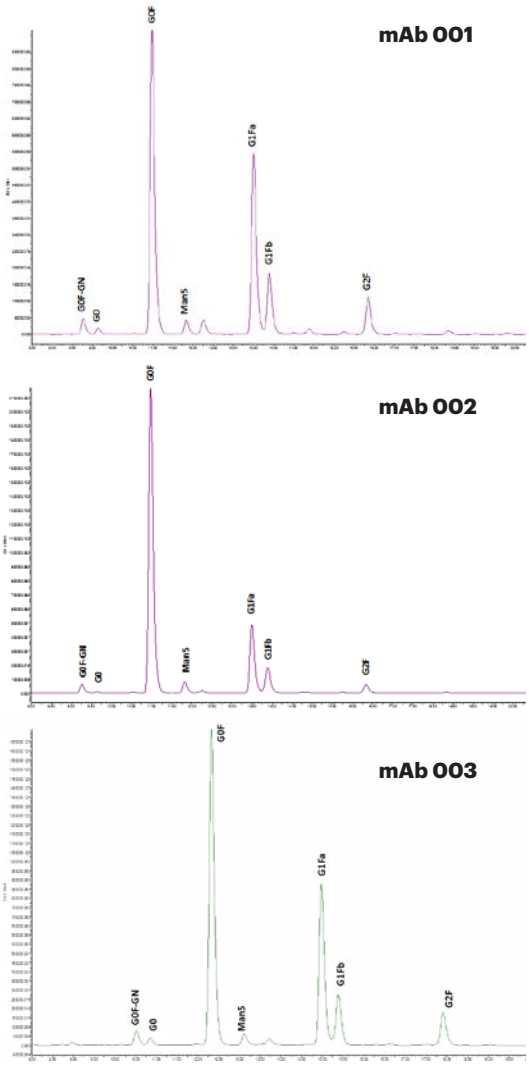

Figure 6. Analysis of *N*-Linked Oligosaccharide of RS by HILIC LC-FLR-MS (2-AB labeling)

Table 7. N-Glycan analysis by HILIC-FLR-MS in-house methods with two labeling systems.

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

TDA: Total Detected Area

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 (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

Table 10. pI values of main charge variants determined by cIEF

Reference Standard	Experimental pI
mAb 001	9.3
mAb 002	7.8
mAb 003	7.8

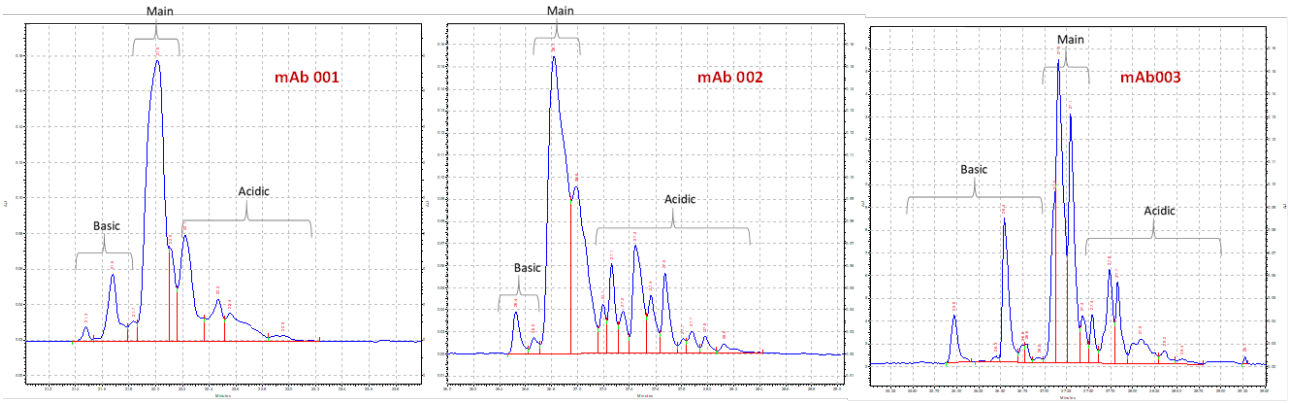

Figure 7. cIEF electropherograms of USP mAbs RS