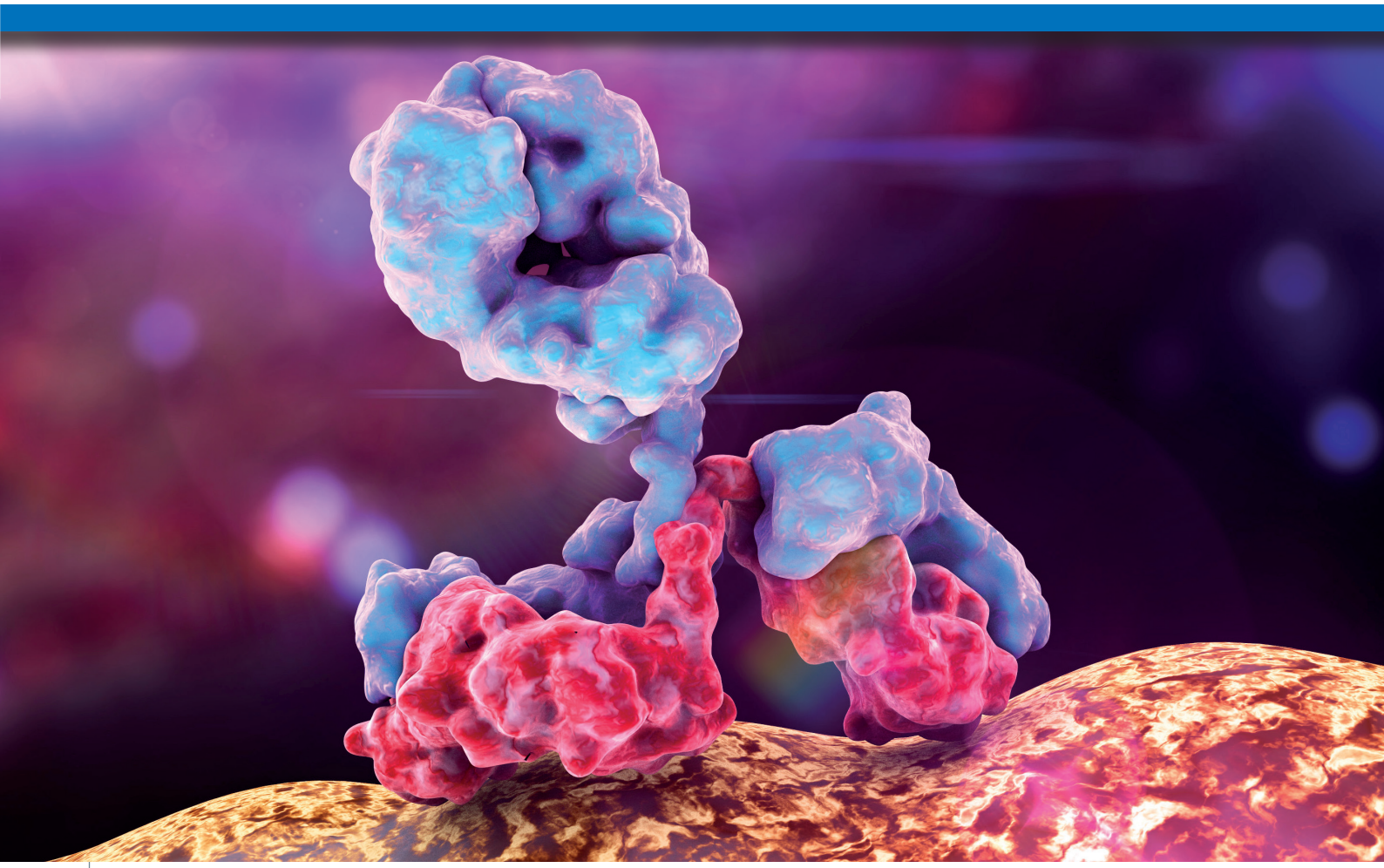

Fully automated glycoform profiling and sequence validation of the NIST reference antibody

Abstract

Two key elements assessed during the analysis of mAb biotherapeutics are the glycosylation profile and the primary sequence. Using the NIST antibody as a model, three complementary approaches, intact, subunit domain and bottom-up, were automated using a new software platform, BioPharma Compass 2.0 (BPC 2.0). These approaches enabled the rapid characterization of the various glycoforms of the antibody and confirmed the sequence and other modifications present with high confidence.

Keywords: Antibody subunits, mAb characterization, biosimilar and innovator development, automated software, maXis II and BioPharma Compass 2.0

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Introduction

There are many different scenarios throughout biopharmaceutical drug discovery and development that require fast, accurate and reliable analysis of antibodies by mass spectrometry, ranging from innovator and biosimilar development to quality control of production.

Since researchers expect fast results with minimal interaction efforts, we developed the BioPharma Compass software platform with an emphasis on simplifying and streamlining automated data acquisition and processing for common analytical workflows. To illustrate the speed with which a large amount of valuable knowledge can be generated and interpreted in BPC 2.0, the NIST reference antibody (1) was measured with the Bruker maXis ultra-high resolution QTOF mass spectrometer using three complementary approaches that have unique capabilities in providing in-depth information quickly (Fig. 1).

Results and Discussion

Intact Mass Screening

Analysis of the intact NIST antibody by UHPLC-MS using only a simple 1 minute desalting LC gradient (total run time 10 minutes) generated valuable information regarding the major glycoforms present in the sample. For example, the main glycoforms G0F, G1F and G2F were annotated, lysine clipping could easily be observed for each glycoform and even the aglycon was detected, indicating a good dynamic range of the analysis. All of these signals are routinely detected with a mass accuracy of < 2 Da, giving a confident overview of the sample's quality and composition in a short timeframe (Fig. 2).

Experimental Details

Platform:	BPC 2.0 software, maXis II UHR-QTOF, Standard flow UHPLC, BEH C4 2.1 x 100 mm 1.7 µm (Waters) or C18+ 2.1 x 100 mm 1.5 µm (Thermo) column (see below), Solvent A 0.1 % FA in water, Solvent B 0.1 % FA in acetonitrile.
Intact mass:	1 µg injected on C4 column heated to 80 °C, 0.5 mL/min, 27-95 % B gradient in 1 min, total run time 10 mins.
Middle-Down:	Digestion with IdeS (FabRICATOR, Genovis) , followed by DTT reduction in guanidine-HCl. 1 µg injected on C4 column heated to 40 °C, 0.2 mL/min, 27-42 % B gradient in 20 min after equilibration to waste for 20 min at 2 % B to remove guanidine-HCl.
Bottom-Up:	Digestion with Trypsin. 4 µg injected on C18 column heated to 40 °C, 0.2 mL/min, 2-40 % B in 45 min, MS 2 Hz, MS/MS 2-5 Hz depending on precursor intensity.

Subunit Domains

A domain-based approach has previously been applied to investigate antibody intact mass discrepancies resulting from sequence variants (2). Here it is used for straight-forward confirmation and relative quantitation of the modifications present on the respective antibody subunit, for example glycation on the LC and pyroglutamylation on the Fd subunit (Fig. 3). Of particular interest, the inlay in Fig. 3 shows that 91.2 % of the mAb, exemplified by the G1F glycoform, had undergone lysine clipping.

The big advantage of the subunit approach is that Bruker's SNAP algorithm calculates monoisotopic molecular weights (in most cases with sub ppm mass accuracy) from isotopic peaks with high isotopic fidelity (True Isotopic Pattern, or TIP™) that were resolved to the baseline (Table in Fig 3). This enables protein modifications to be simply and accurately quantified, and even allows deamidation events to be studied on the domain level.

Both expected and unexpected protein modifications can be confidently assigned in BPC 2.0, and are further validated by overlaying the theoretical isotope pattern.

Bottom-Up Analysis

Sequence validation of antibodies requires the fragmentation of all available peptides in order to achieve near 100 % sequence coverage, and the fragment spectra generated must be of very high quality in order to provide fragment peak evidence (fragment coverage) for each individual amino acid.

The NIST antibody was digested with trypsin only and the peptides were separated using a relatively short 45 min gradient. Fragmentation was triggered using an Instant Expertise™ auto MS/MS acquisition method and yielded 99.5 % sequence coverage for the light chain (LC) in a single experiment (Fig. 4).

This result was obtained with MS/MS fragment spectra that exhibited excellent detection of low and high mass fragments simultaneously, which resulted in 96.2% fragment coverage of the NIST LC.

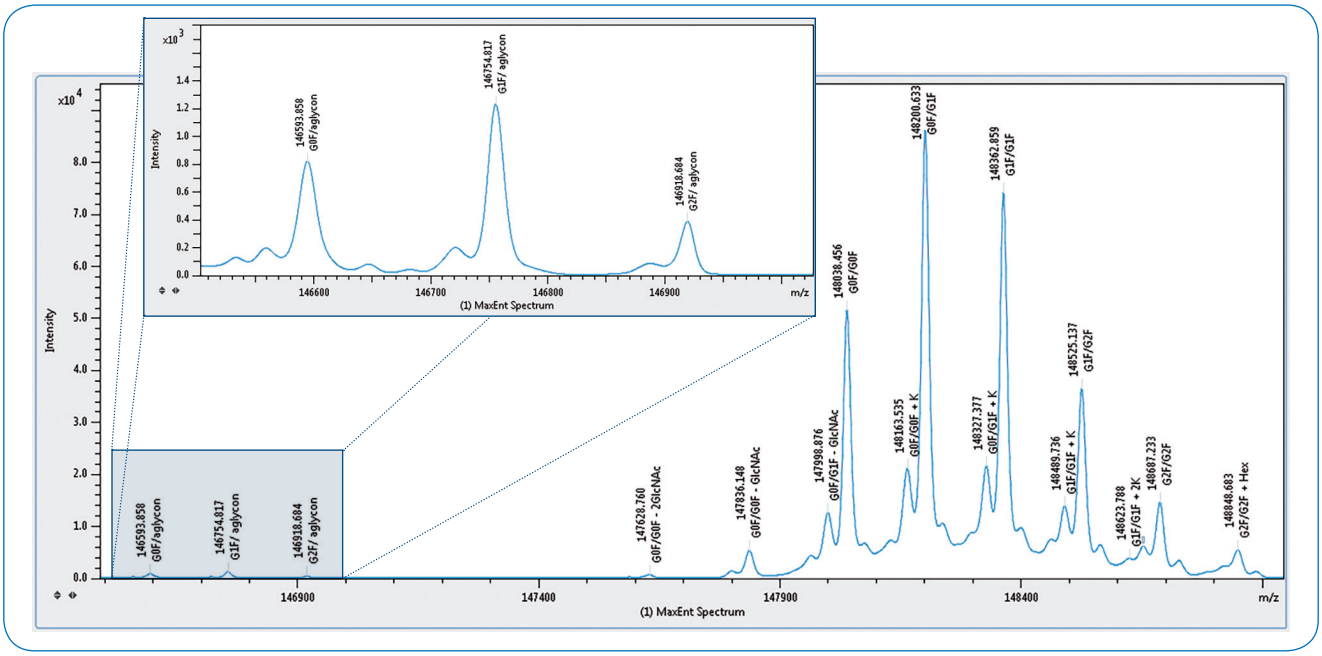
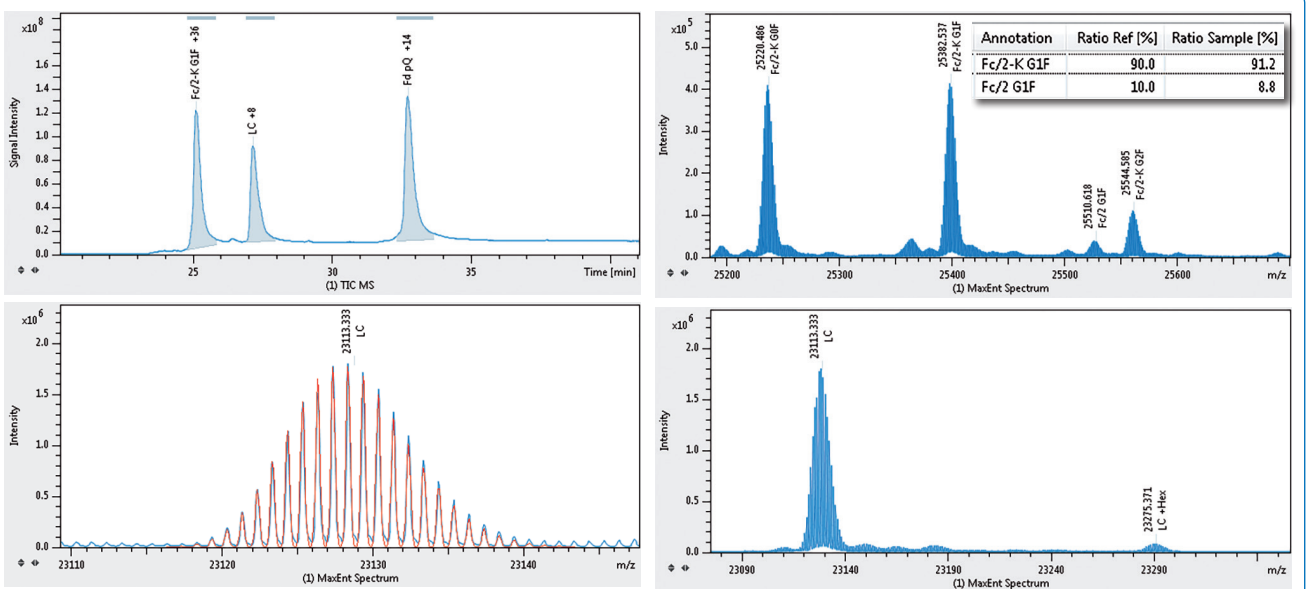
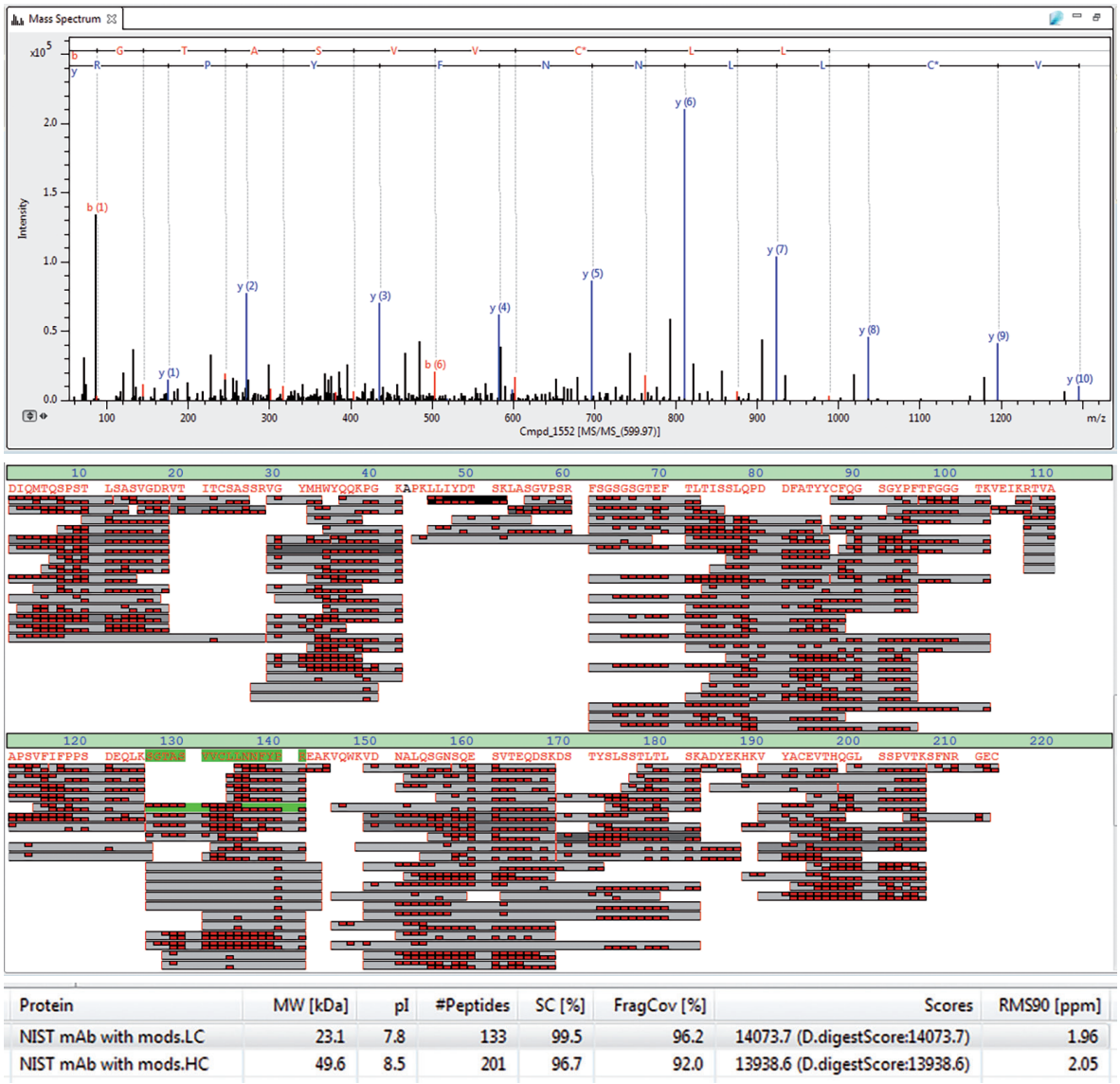


Fig. 2: Intact mass screening of NIST antibody. Maximum entropy deconvoluted spectrum shows glycoforms detected across > 2 orders of magnitude. Insert shows low abundant aglycon forms in more detail.



Annotation	Mr Ref	Mr Sample	Δ Mr [ppm]	Δ Mr [Da]
LC	23113.3042	23113.3329	1.24	0.0287
Fd pQ	25672.8066	25672.8215	0.58	0.0149
Fc/2-K G1F	25382.5162	25382.5369	0.81	0.0207
Fc/2-K G0F	25220.4634	25220.4863	0.91	0.0229
Fc/2-K G2F	25544.5690	25544.5848	0.62	0.0158
LC +Hex	23275.3570	23275.3706	0.58	0.0136
Fd pQ +Hex	25834.8594	25834.8540	-0.21	-0.0054
Fc/2 G1F	25510.6112	25510.6178	0.26	0.0066

Fig. 3: Subunit domain analysis of NIST antibody. Left from top, LC-MS TIC chromatogram, NIST LC acquired isotopic pattern (blue) overlaid with theoretical (red), table of results (abridged). Right from top, maximum entropy deconvoluted spectra for NIST Fc/2 (insert shows relative quantitation of lysine clipping), LC and Fd subunits.



Protein	MW [kDa]	pI	#Peptides	SC [%]	FragCov [%]	Scores	RMS90 [ppm]
NIST mAb with mods.LC	23.1	7.8	133	99.5	96.2	14073.7 (D.digestScore:14073.7)	1.96
NIST mAb with mods.HC	49.6	8.5	201	96.7	92.0	13938.6 (D.digestScore:13938.6)	2.05

Fig. 4: Bottom up analysis of NIST antibody showing near 100% sequence coverage. From top, example MS/MS spectrum, sequence coverage map and an excerpt from the BPC 2.0 results table

Conclusion

BioPharma Compass 2.0 and the maXis II were used for the automated assessment of the NIST antibody using three complementary approaches, intact, subunit domain and bottom-up. This combination provided fast, accurate and in-depth information for antibody analysis using a single software platform. BPC 2.0 was developed for maximum ease-of-use whilst retaining the experimental flexibility required by today's biopharmaceutical analytical laboratory. The software also supports 21 CFR Part 11 compliant work.

The particular advantages of the described approaches are:

Intact mass: Fast screening for expected glycoforms including low abundant species such as the aglycan and early detection of antibody processing artefacts.

Subunit domain: Accurate mass analysis of antibody subunits on the sub ppm level, glycoform quantitation and further localization of sequence deviations on the Fd, Fc/2 or LC domains (2).

Bottom up: Near 100% sequence and fragment coverage for sequence validation from a single experiment.

BioPharma Compass 2.0

Automated in-depth antibody analysis – made simple

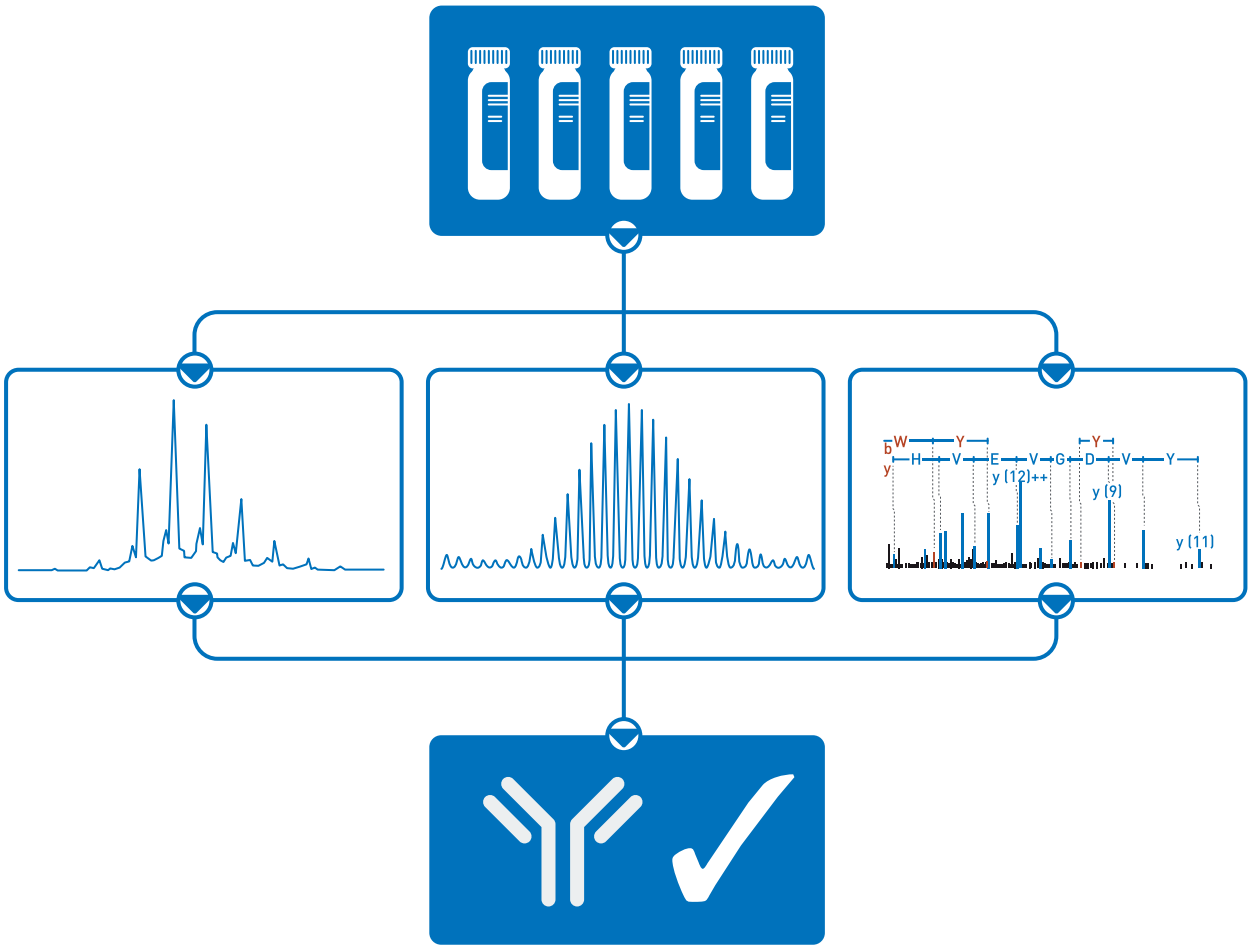


Fig. 1: Graphical abstract, showing the use of BioPharma Compass 2.0 for automated antibody characterization using intact, subunit domain and bottom-up approaches. The software also contains other features not shown here, including workflows for MALDI and ETD, and a Regulatory Toolkit.

Learn More

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References

1. **Formolo T et al. (2015), Determination of the NISTmAb Primary Structure, in: State-of-the-Art and Emerging Technologies for Therapeutic Monoclonal Antibody Characterization Volume 2. Biopharmaceutical Characterization: The NISTmAb Case Study, Chapter 1, pp 1–62, ACS Symposium Series, Vol. 1201; Chapter DOI: 10.1021/bk-2015-1201.ch001.**
2. **Ayoub D et al. Correct primary structure assessment and extensive glyco-profiling of cetuximab by a combination of intact, middle-up, middle-down and bottom-up ESI and MALDI mass spectrometry techniques. MAbs. 2013;5(5):699-710. doi: 10.4161/mabs.25423.**

Some of the work reported here was undertaken as part of a project for the Consortium for Top-Down Proteomics: "Structural Analysis of Monoclonal Antibodies by Top-Down Mass Spectrometry: An Intra-Laboratory Study".

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