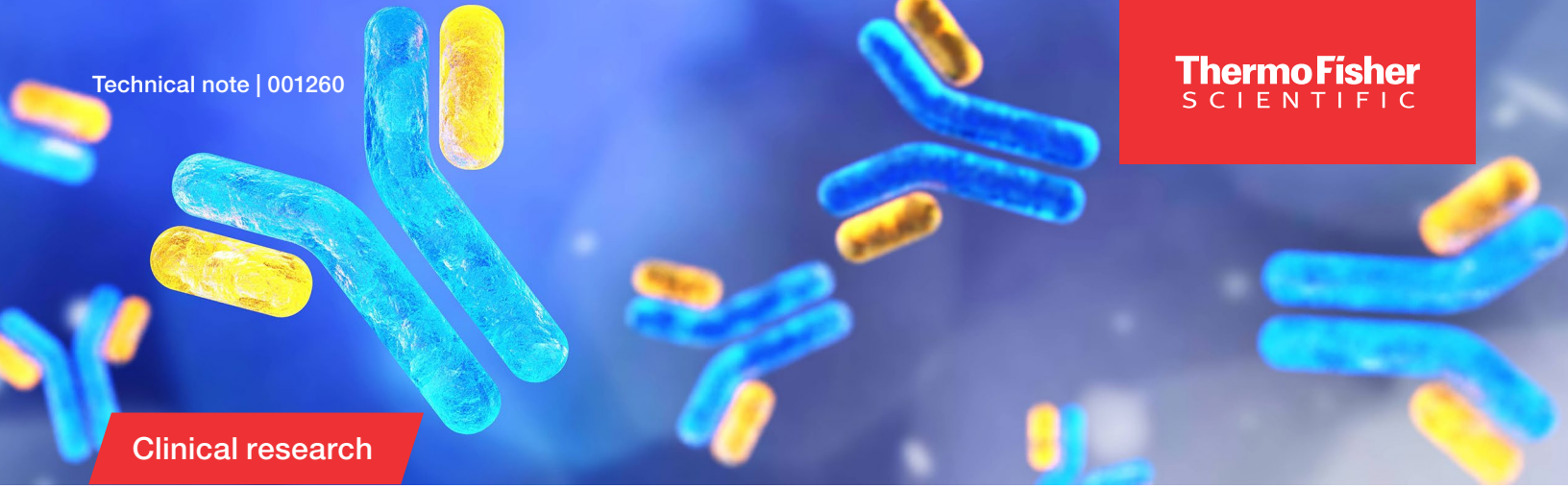

Monitoring the intact light chain of therapeutic monoclonal antibodies in human serum using an Orbitrap Exploris 240 mass spectrometer for clinical research

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Keywords

Monoclonal antibodies, therapeutic drug monitoring, mAbs, TDM, subunit, middle-up, Orbitrap Exploris 240 MS, Vanquish Flex UHPLC, MAbPac RP column, TraceFinder software, Protein L purification, magnetic beads, LC-MS, mass spectrometry, adalimumab, bevacizumab, camrelizumab, daratumumab, golimumab, rituximab, nivolumab, pembrolizumab

Application benefits

- Targeted quantitation of intact protein using HRAM Orbitrap™ mass spectrometry for clinical research
- Low volume sampling of 10 µL per analysis with <1.5 hours of sample preparation
- Minimized sample loss by seamless sample treatment on the beads
- Single software platform from data acquisition to data processing/reporting

Goal

To develop a light chain quantitation workflow to measure the concentration of monoclonal antibodies in human serum using Protein L purification and the Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer

Introduction

Each year, more monoclonal antibodies (mAbs) are approved by regulatory agencies to treat a wide range of diseases with an expected market value of over $200 billion by 2026.[1,2] The advantage of using therapeutic mAbs lies in their inherent specificities for recognizing and counteracting foreign substances (so-called antigens). Due to their molecular complexity, including post-translational modifications, biopharmaceutical companies have embraced many advanced analytical techniques such as mass spectrometry to better characterize and quantify mAbs during the development stages. In clinical testing, the presence of endogenous immunoglobulins in patients' samples with nearly identical structures to therapeutic mAbs adds an additional challenge to the accurate quantitation of therapeutic mAbs. Accordingly, mass spectrometry has also gained substantial popularity for therapeutic mAb monitoring (i.e., TDM of mAb) in clinical laboratories due to its great versatility to detect both tryptic peptides and intact light and heavy chains quantitatively.[3,4]

Here we present the intact light chain quantitation approach for measuring concentrations of therapeutic mAbs in human serum using the Orbitrap Exploris 240 MS for clinical research. One of the benefits of intact light chain quantitation is a simplified workflow with faster sample preparation compared to peptide quantitation. Additionally, it can provide a solution for mAbs that contain limited or no signature tryptic peptides and are not possible to differentiate from endogenous IgG. Protein L magnetic beads were used for the selective purification of antibodies containing kappa light chains. This way, endogenous lambda light chains from human serum are not captured, which can improve the purity of isolated antibodies and potentially be applied to other modality antibodies or Fab fragments containing kappa light chains.

Experimental

Sample preparation in human serum

Six mAbs evaluated in this study included adalimumab, bevacizumab, camrelizumab, daratumumab, golimumab, and rituximab, while two additional mAbs, nivolumab and pembrolizumab, were used as internal standards (IS). The experimental workflow is described in Figure 1, and assignments of two IS are listed in Table 1. Nivolumab (N) was assigned to mAbs with light chain masses >23.5 kDa while pembrolizumab (P) was assigned to those <23.5 kDa. All mAbs were diluted to 1 mg/mL based on their initial concentration and then to different concentrations of mAb stock solutions according to Table 2. These stock solutions were diluted with a normal pool of human serum using the same dilution factor to generate the final concentration points of the calibration curve ranging from 1 µg/mL to 100 µg/mL. The IS mAbs were spiked to the calibration curve samples to make a final concentration of 100 µg/mL.

Table 1. List of target mAbs evaluated in this report and assignment of nivolumab (N) and pembrolizumab (P) as IS per target mAb

mAb	Light chain mass	IS assignment
Rituximab	23042.3	P
Nivolumab (N)	23359.5	
Daratumumab	23369.6	P
Adalimumab	23397.6	P
Bevacizumab	23436.4	P
Golimumab	23547.6	N
Camrelizumab	23720.5	N
Pembrolizumab (P)	23729.8	

Table 2. Calibration curve generation of target mAbs

Serial dilution from C0 to C7	Final concentration of mAb (µg/mL)	Concentration of mAb stock solution (µg/mL)
C0	0	0
C1	1	5
C2	2	10
C3	5	25
C4	10	50
C5	20	100
C6	50	250
C7	100	500

Antibody purification

Thermo Scientific™ Pierce™ Protein L magnetic beads (P/N 88850) were used for the antibody purification following the published procedures (Pub. No. MAN0011858) with minor modifications. The binding/wash buffer was Tris-buffered saline using Thermo Scientific™ BupH™ Tris Buffered Saline Packs (P/N 28379) containing 0.05% Tween™-20 detergent (Fisher BioReagents™, P/N BP337-500). An aliquot of 50 µL of Protein L magnetic beads

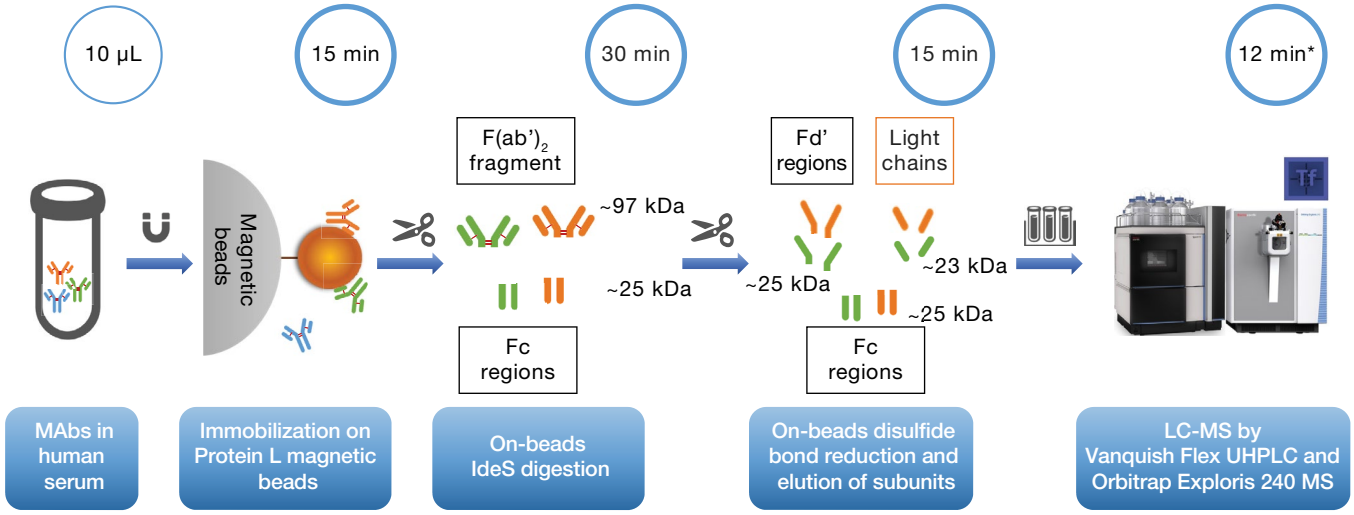

Figure 1. Experimental workflow illustrating sample preparation in human serum, antibody capture on Protein L magnetic beads, IdeS digestion, disulfide bond reduction, and LC-MS analysis

was dispensed into a 1.5 mL microcentrifuge tube followed by adding 200 µL of the binding/wash buffer. The beads were mixed by gentle pipetting multiple times without vortexing. The sample tube was then placed on a magnet and the supernatant was removed. The magnetic beads were preconditioned one time with 500 µL of the binding/wash buffer. An aliquot of 10 µL of the samples was diluted with 490 µL of the binding/wash buffer. The diluted samples were added to the preconditioned beads and incubated for 15 minutes at room temperature with rotation at a fixed speed of 18 rpm. The sample tubes were placed on the magnet and the supernatant was removed. An aliquot of 500 µL of the binding/wash buffer was added and mixed by gentle pipetting multiple times. This wash step was repeated three times. After adding the third binding/wash buffer, the beads were transferred to a new 1.5 mL microcentrifuge tube to avoid any impact from residual surfactant left on the tube wall. After removing the third supernatant, 200 µL of phosphate-buffered saline (Thermo Scientific™ BupH™ Phosphate Buffered Saline Packs, P/N 28372) were added for the final wash. The tube was placed on a magnet and the supernatant was removed.

On-beads IdeS digestion and disulfide bonds reduction

The purified samples were directly subjected to IdeS digestion on the beads following the protocol provided by the vendor (FabRICATOR™ Ides, Genovis). The beads were resuspended with 95 µL of LC-MS grade water and 5 µL of 10 units/µL of IdeS were added. The samples were then incubated for 30 minutes at 37 °C with mixing at 1,200 rpm. After incubation, an aliquot of 100 µL of Thermo Scientific™ Pierce™ 8 M Guanidine-HCl Solution (P/N 24115) was added to make a final concentration of 4 M followed by the addition of Thermo Scientific™ Bond-Breaker™ TCEP Solution, neutral pH (P/N 77720) at a final concentration of 10 mM. The samples were mixed by gentle pipetting and incubated for 15 minutes at 57 °C with mixing at 1,200 rpm. During this step, proteins were eluted so no additional elution steps were necessary. After cooling down for 5 min at room temperature, the tube was placed on a magnet and the eluate was transferred to an Amicon™ Ultra-0.5 centrifugal filter device (10k MWCO). The samples were desalted and concentrated to 100 µL. Prior to LC-MS analysis, the samples were diluted two-fold with 40% mobile phase B to make a final percentage of 20% mobile phase B as a loading condition.

Liquid chromatography

LC separation was performed on a Thermo Scientific™ Vanquish™ Flex Binary UHPLC system with a Thermo Scientific™ MAbPac™ RP HPLC column (2.1 × 50 mm, 4 µm, P/N 088648). Separation conditions and LC gradients are listed in Table 3.

Table 3. LC conditions

Parameter	Separation conditions			
Mobile phase A	0.1% formic acid and 0.02% trifluoroacetic acid in water			
Mobile phase B	0.1% formic acid and 0.02% trifluoroacetic acid in 10:20:70 water: isopropanol: acetonitrile (v/v/v)			
Flow rate (mL/min)	0.4			
Column temperature (°C)	80 (Still air)			
Injection volume (µL)	10			
LC gradient	Time (min)	%A	%B	Curve
	0.0	80	20	5
	0.5	80	20	5
	0.7	68	32	5
	8.5	60	40	5
	9.2	20	80	5
	9.7	20	80	5
	9.8	80	20	5
	12.0	80	20	5

Mass spectrometry

MS analysis was performed on a Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer with BioPharma option installed. MS parameters are described in Table 4.

Table 4. MS parameters

MS global parameters	
Application mode	Intact protein
Pressure mode	Low pressure
Source type	Heated electrospray ionization (H-ESI)
Polarity	Positive
Spray voltage (V)	3,800
Sheath gas (Arb)	25
Aux gas (Arb)	5
Sweep gas (Arb)	2
Ion transfer tube temp. (°C)	320
Vaporizer temp. (°C)	150
Divert valve A	0.0 min: position 1-6 (waste) 1.0 min: position 1-2 (MS) 9.5 min: position 1-6 (waste)
MS scan parameters	
Orbitrap resolution	120,000
Scan range (*m/z*)	700–2,000 for intact mass confirmation 1,000–1,400 for targeted quantitation
RF lens (%)	60
AGC target	Standard
Maximum injection time mode	Custom
Maximum injection time (ms)	200

Data acquisition and processing

For intact mass analysis, Thermo Scientific™ BioPharma Finder™ 4.1 software was used. Sequences of three subunits were generated as separate databases using the sequence manager prior to intact mass analysis. Since glycosylation is present in the Fc/2 region, two major glycan structures, A2G0F and A2G1F, were added as variable modifications to the Fc/2 sequence. Most of the default parameters from the Xtract deconvolution algorithm remained unchanged, except the target average spectrum window from sliding windows was set to between 0.1 and 0.15 min and deconvolution mass tolerance was set to 10 ppm. The *m/z* and RT ranges were also modified according to the method.

For targeted quantitation, data acquisition and processing were performed using Thermo Scientific™ TraceFinder™ 5.1 software. Table 5 includes a list of *m/z* values used for peak extraction using a mass accuracy of 10 ppm so the peak areas from a total of 12 *m/z* values were summed to quantify each mAb light chain. Each data point of the calibration curve was analyzed in triplicate and then fitted with % accuracy between 80 and 120, % RSD < 15, % CV < 15, and R^2 > 0.99 to determine the limits of quantitation (LOQ) for each mAb light chain. The limits of detection (LOD) and linear range were also determined.

Table 5. List of top 3 intense *m/z* values from each isotopic distribution of 4 most intense charge states used for targeted quantitation

mAb	Charge state	Top 3 intense *m/z* values from each charge state		
Adalimumab	+23	1018.8671	1018.9052	1018.9486
	+22	1065.0822	1065.1277	1065.1733
	+21	1115.7527	1115.8000	1115.8480
	+20	1171.4895	1171.5400	1171.5900
Bevacizumab	+23	1020.5070	1020.5505	1020.5939
	+22	1066.8931	1066.9387	1066.9840
	+21	1117.6017	1117.6496	1117.6968
	+20	1173.4381	1173.4885	1173.5384
Camrelizumab	+23	1013.3355	1013.3789	1013.4225
	+22	1059.3502	1059.3951	1059.4407
	+21	1109.6995	1109.7472	1109.7949
	+20	1165.1340	1165.1843	1165.2341
Daratumumab	+23	1017.6422	1017.6861	1017.7297
	+22	1063.8526	1063.8984	1063.9438
	+21	1114.4171	1114.4644	1114.5124
	+20	1170.1371	1170.1871	1170.2378
Golimumab	+23	1025.3851	1025.4283	1025.4714
	+22	1071.9018	1071.9472	1071.9931
	+21	1122.8996	1122.9445	1022.9925
	+20	1179.0413	1179.0920	1179.1415
Rituximab	+23	1002.6320	1002.6754	1002.7188
	+22	1048.1598	1048.2053	1048.2507
	+21	1098.0242	1098.0719	1098.1196
	+20	1152.8725	1152.9223	1152.9725
Nivolumab	+23	1017.2065	1017.2497	1017.2935
	+22	1063.3518	1063.3975	1063.4427
	+21	1113.9395	1113.9874	1114.0346
	+20	1169.6365	1169.6861	1169.7359
Pembrolizumab	+23	1033.3051	1033.3460	1033.3919
	+22	1080.2277	1080.2731	1080.3193
	+21	1131.5711	1131.6189	1131.6666
	+20	1188.0993	1188.1495	1188.2003

Results and discussion

Optimization of workflow

One of the challenges in mAb monitoring is the limited availability of heavy isotope-labeled versions of mAbs. A novel strategy was employed in this report to use alternative mAbs as internal standards (IS). After investigation, nivolumab and pembrolizumab were selected based on their light chain masses as shown in Table 1. Since this approach extracted *m/z* values of different charge states, the *m/z* values of the IS and the target mAb should occupy a different mass space. The ideal IS mass should have at least 200 Da difference from the target mAb, which will result in ≥6 *m/z* intervals (charge state ≤ +30) without overlapping with the target mAb. With this consideration, nivolumab was assigned to mAbs with their light chain masses >23.5 kDa, while pembrolizumab was assigned to those <23.5 kDa.

The Orbitrap Exploris 240 mass spectrometer fully resolved isotopic clusters of different charge states of three subunits by operating at a resolution >120k. Also, the intact protein application mode with low pressure provided more sensitive and robust detection of the intact subunits (data not shown). Figure 2 shows isotopic clusters of bevacizumab light chain at a charge state of 21 using different resolutions on the Orbitrap analyzer, from 15k to 240k. Since endogenous IgGs are also captured and detected over similar mass distributions with the target mAbs, the Orbitrap Exploris 240 mass spectrometer when operated at >120k resolution significantly improves accurate identification and quantitation of the intact subunits in a complex matrix.

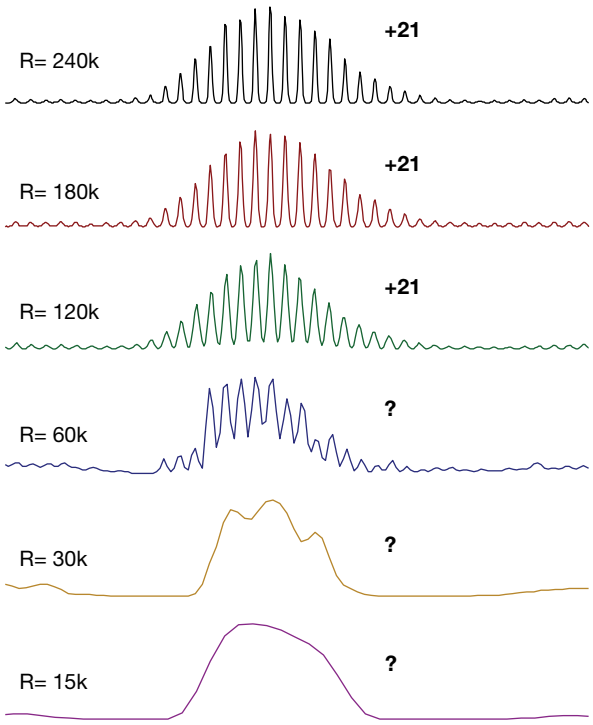

Figure 2. Isotopic clusters of bevacizumab light chain at a charge state of 21 using different resolutions on Orbitrap MS

For antibody purification, Protein L magnetic beads were chosen among different purification techniques due to the fact that Protein L ligands selectively and strongly bind to mouse and human antibodies containing kappa light chains. Since the majority of therapeutic mAbs contain kappa light chains, it can improve the purity of the final samples by not capturing endogenous lambda chains. Although it still captures endogenous kappa chains, the Protein L still resulted in a higher purification yield compared to other techniques tested including Protein G and A (data not shown).

Further optimization was performed including incorporating subunit digestion and disulfide bond reduction on the beads. The benefits of implementing on-beads sample treatment include minimizing sample loss and bypassing additional elution and buffer or volume adjustment. Furthermore, using a denaturing reagent ensures a complete disulfide bond reduction and elution of all subunits from the beads. It generated a uniform structure of intact subunits as a single peak as shown in Figure 3A, representing excellent separation of three subunits, Fc/2, light chain (indicated as LC), and Fd', without observation of any non or partially reduced species. Figure 3B shows isotopic distributions of observed charge states for each subunit. Using BioPharma Finder software, these isotope envelopes were deconvoluted to each intact molecular mass and accurately assigned with a mass accuracy of ≤10 ppm as seen in Figure 3C. For the Fc/2 chain, the major protein isoform was assigned with A2G0F glycan as it contains one N-linked glycosylation site.

For targeted quantitation, the full MS scan range was adjusted to target *m/z* values from 1,000 to 1,400 without target mass inclusion as shown in Figure 3D. As shown in Table 5, the top three intense *m/z* values from the four most abundant charge states of the light chain were added to TraceFinder software for peak extraction so the peak areas from a total of 12 *m/z* values were summed for the light chain quantitation using a mass tolerance of 10 ppm.

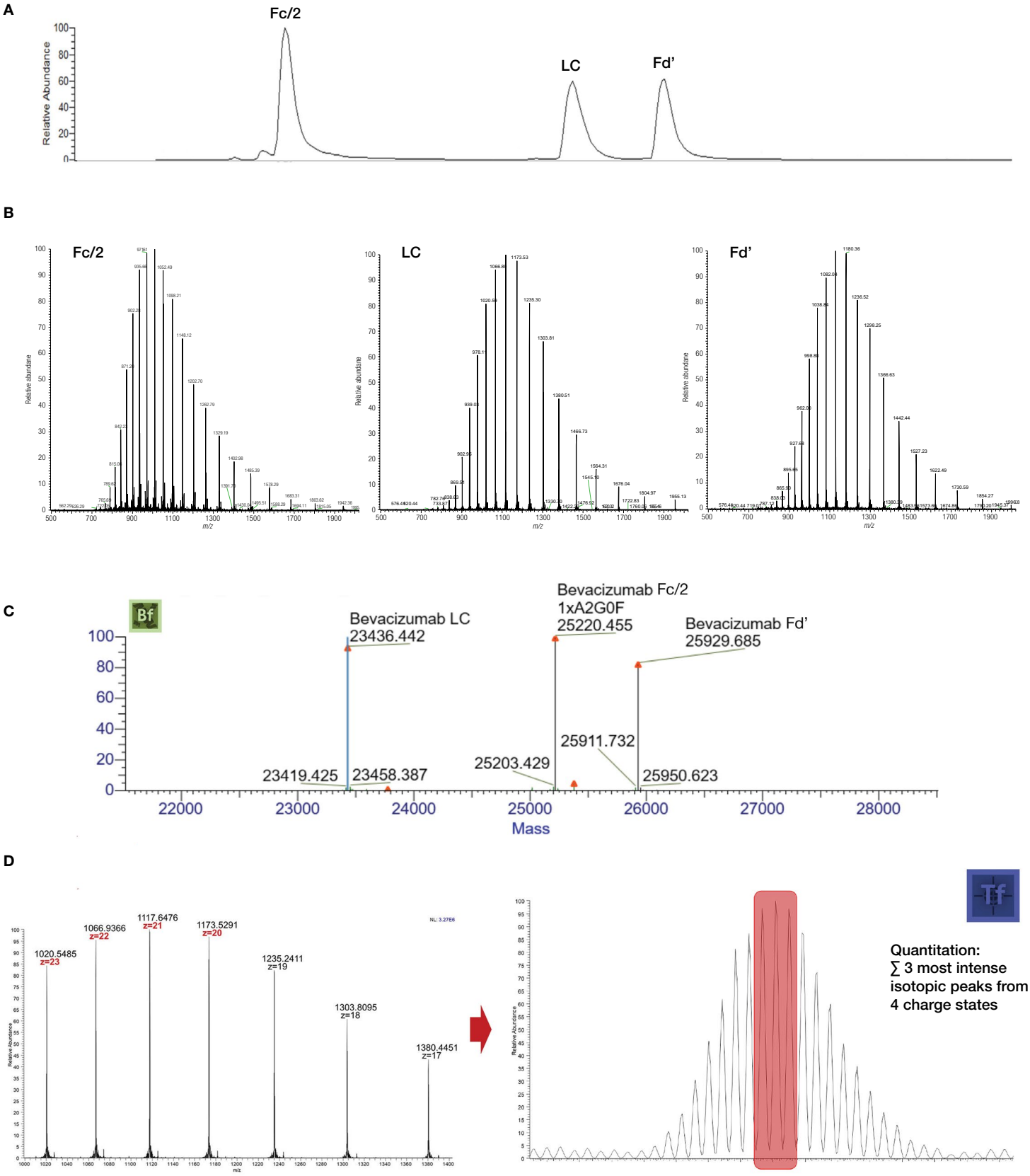

Figure 3. Retention times of bevacizumab subunits, Fc/2, LC (light chain), and Fd' (A), full MS spectrum under each subunit (B), assigned subunits and their intact masses from BioPharma Finder software (C), and targeted quantitation strategy by scanning narrow MS range and extracting intense ions using TraceFinder 5.1 software (D)

Evaluation of analytical performance

All six mAbs were successfully monitored with the optimized workflow. Figure 4 shows the representative extracted ion chromatograms (XICs) of each mAb light chain. Table 6 lists LOD, LOQ, linear dynamic range, and R^2 values for each mAb. Excellent linearity was observed for all the mAbs with R^2 values higher than 0.99 as shown in Table 6 and Figure 5A. Also, Figure 5B shows the peak areas of the internal standards for golimumab (nivolumab) and bevacizumab (pembrolizumab) from the calibration curve analyses. The % RSD of the peak areas was less than 15%, which supports the reproducibility of the entire process from sample preparation to LC-MS analysis. Reproducible retention times were observed over all the calibration curve generations as shown in Figure 6A. The variation of detected retention time of two IS mAbs was determined to be less than ±0.05 minutes. An example of overlaid XICs is depicted in Figure 6B, pembrolizumab detection at *m/z* 1033.3051 with a mass accuracy of 10 ppm from 24 injections in the rituximab calibration. LOQs were determined to be between 1 to 5 µg/mL of the mAb concentration in serum as listed in Table 6.

Evaluation of column reproducibility

Two columns from two different lots were purchased and evaluated in the following order: column 1 – lot 02020052 (Serial No. 002561), column 2 – lot 02020103 (Serial No. 002598), column 3 – lot 02020052 (Serial No. 002576), and column 4 – lot 02020103 (Serial No. 002587). The same samples were analyzed with the same mobile phases on consecutive days. Each column was conditioned equally prior to the column evaluation. As shown in Table 7 and Figure 7, peak areas were not significantly different, showing a less than 20% difference between the maximum and minimum peak areas. The reproducible retention times were also observed within the same lots with <0.05 minutes difference, while the retention times between the different lots showed a slight shift with <0.2 minutes difference. Although it was not a full validation of the column reproducibility, this report demonstrates that the MAbPac column generates coherent intact protein data for clinical research including reproducible retention times and peak areas.

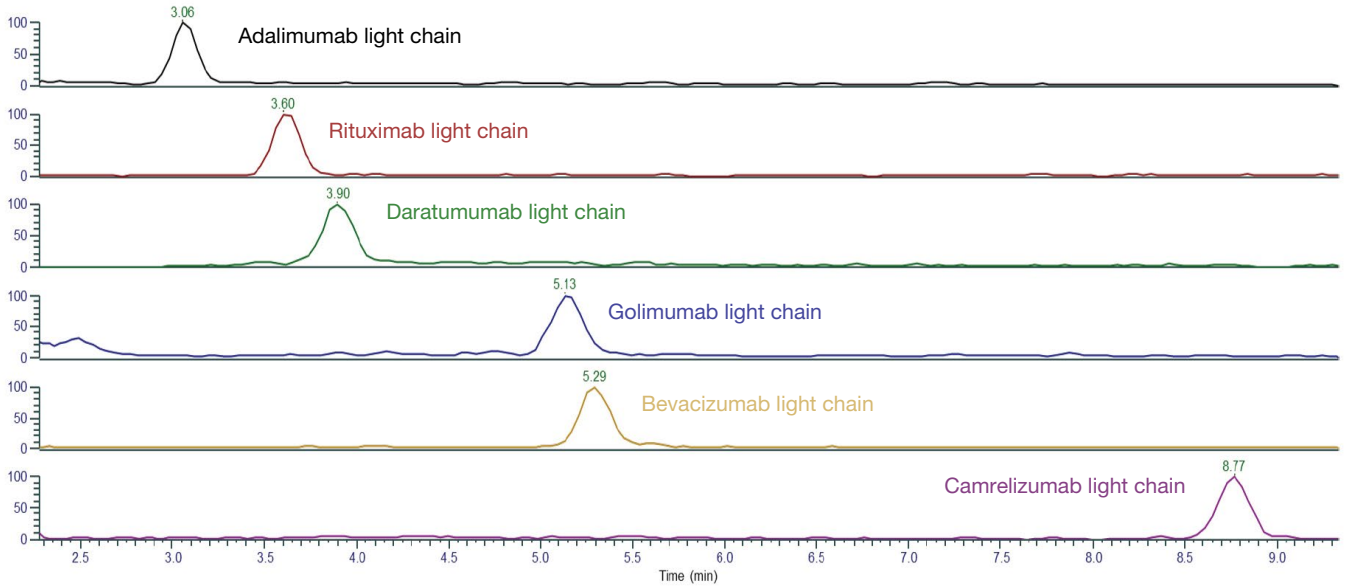

Figure 4. Representative XICs of target mAb light chain

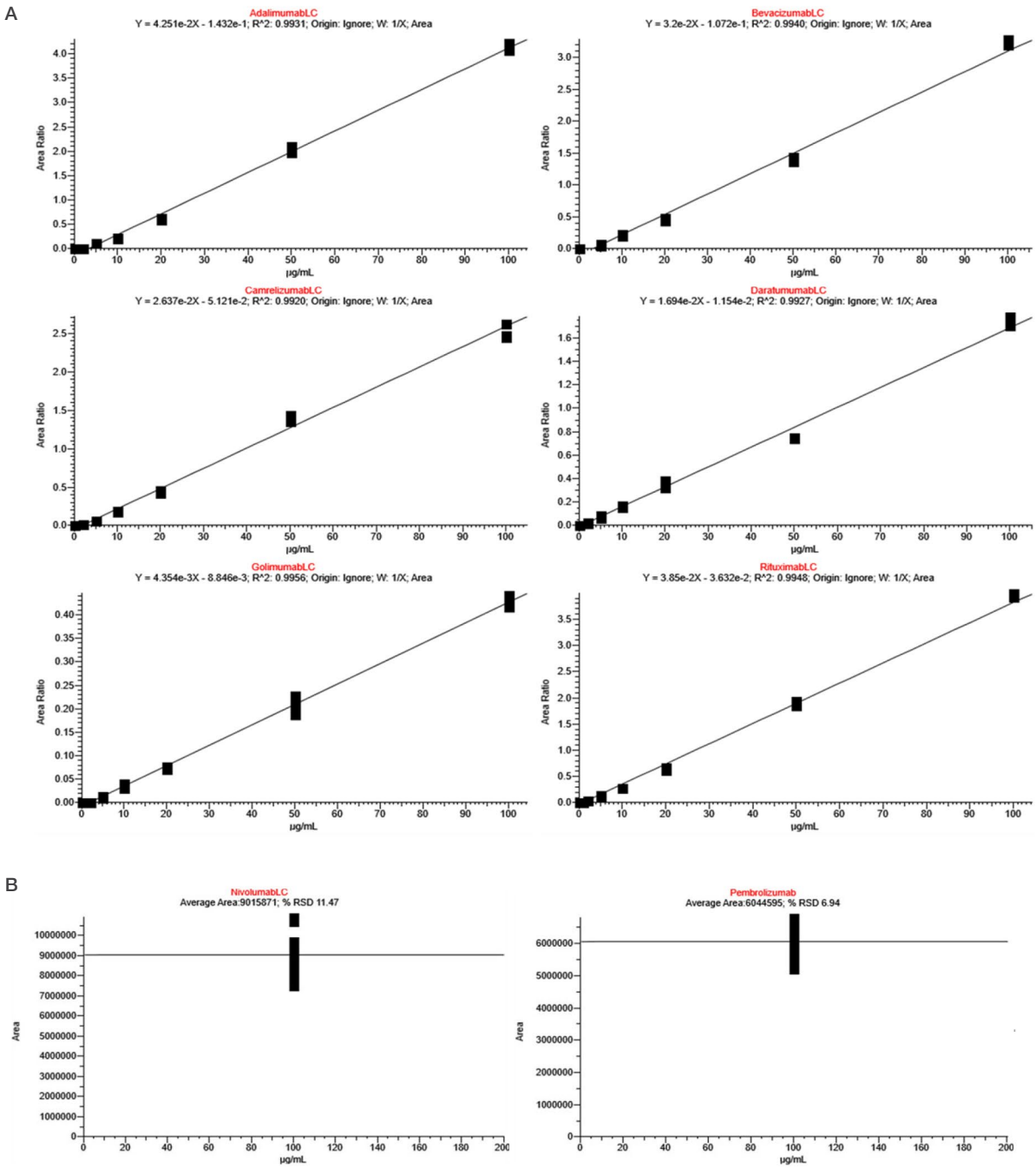

Figure 5. Calibration curves of six target mAbs (A) and peak areas of two IS mAbs, nivolumab and pembrolizumab, from golimumab and bevacizumab calibration curve analyses, respectively (B)

Table 6. Determined analytical properties of the method from the calibration curve including LODs, LOQs, linear range, and R^2 values.

mAb	LOD (µg/mL)	LOQ (µg/mL)	Linear range (µg/mL)	R^2
Adalimumab	2	2	2–100	0.9931
Bevacizumab	5	5	5–100	0.9940
Camrelizumab	2	2	2–100	0.9920
Daratumumab	2	2	2–100	0.9927
Golimumab	2	5	5–100	0.9956
Rituximab	1	1	1–100	0.9948

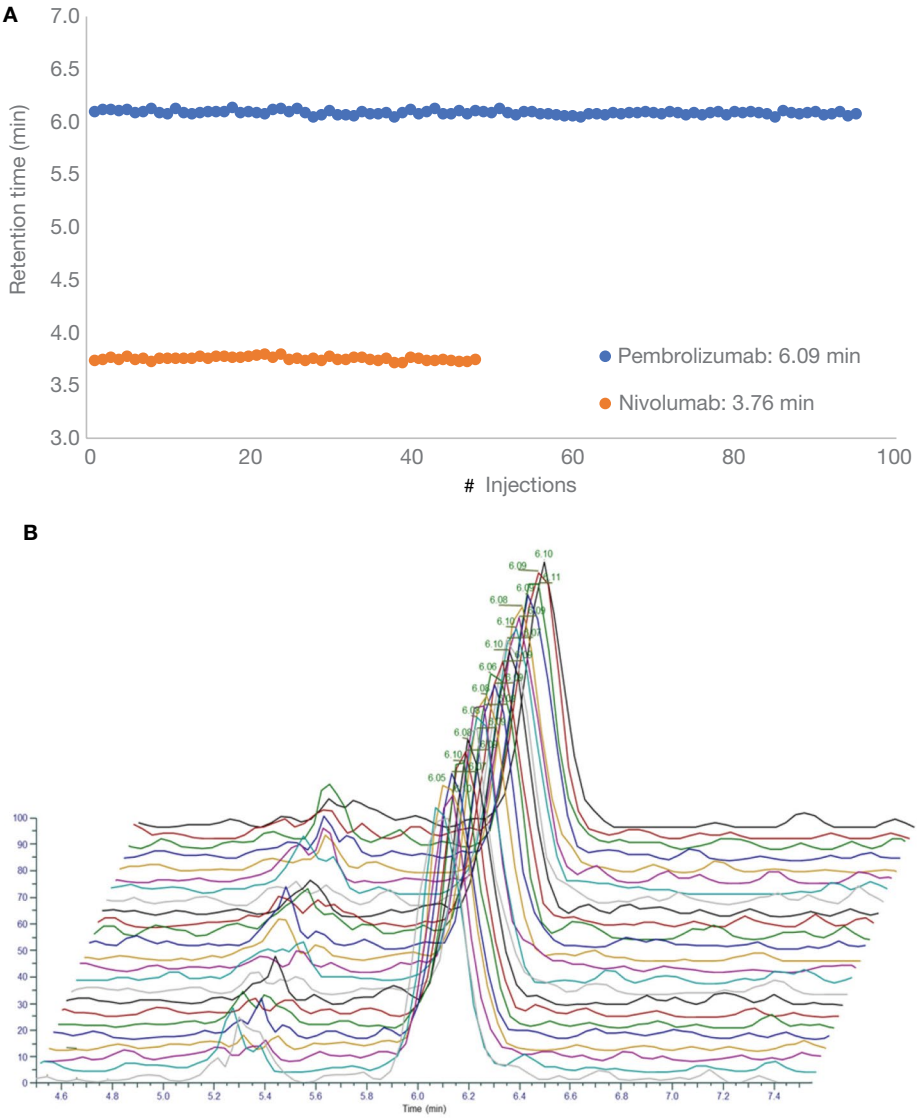

Figure 6. Observed retention times of two IS mAbs over the entire calibration curve analysis (A) and overlaid XICs of pembrolizumab at *m/z* 1033.3051 with mass accuracy of 10 ppm from 24 injections in the rituximab calibration (B)

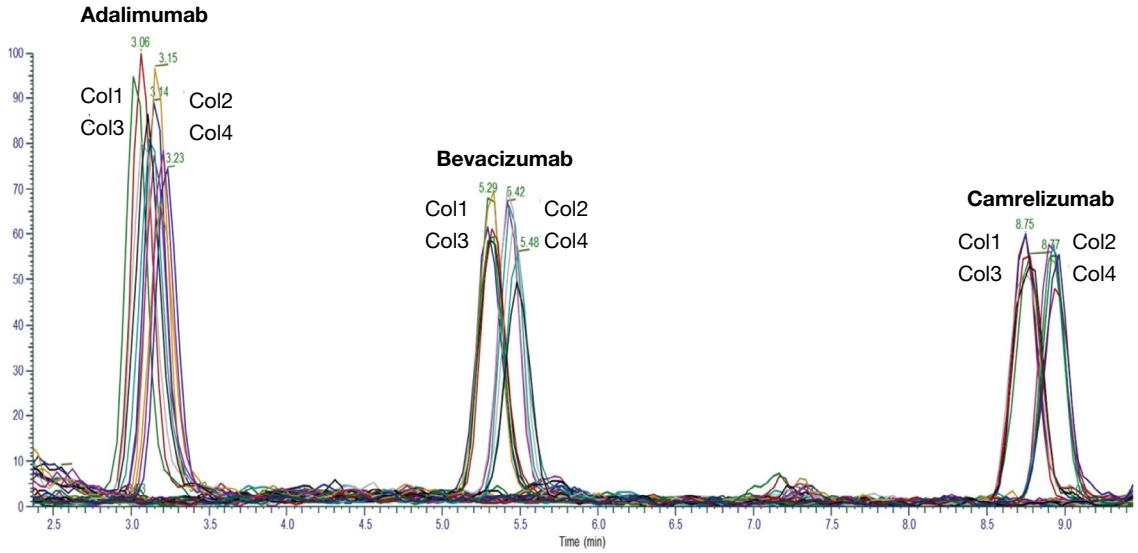

Figure 7. Overlaid XICs of adalimumab, bevacizumab, and camrelizumab from column reproducibility evaluation

Table 7. Results of column reproducibility evaluation

	Adalimumab	Bevacizumab	Camrelizumab	Daratumumab	Golimumab	Rituximab
Average RT (minutes)	3.12	5.38	8.85	3.98	5.20	3.67
RT |Max - Min| (minutes)	0.15	0.18	0.18	0.14	0.16	0.15
Average peak area	3.52E+06	3.13E+06	3.12E+06	1.92E+06	1.33E+06	2.98E+06
% Peak area difference |Max - Min|	19%	20%	16%	14%	9%	13%

Conclusion

In this report, we successfully developed the workflow for mAb monitoring in human serum using the Orbitrap Exploris 240 mass spectrometer for clinical research. The intact protein application mode with low pressure provides the best performance for the intact light chain analysis, which is accessible by installing the BioPharma Option. It should be noted that the BioPharma Option is available for the Orbitrap Exploris 240 and Orbitrap Exploris 480 mass spectrometers. Thus, implementing this workflow on an Orbitrap Exploris 120 mass spectrometer needs a method modification regarding the availability of different application modes. The Protein L magnetic beads were applied to capture the kappa light chains of the mAbs, and on-beads sample treatment was introduced to minimize unnecessary steps and sample loss and maintain the sample integrity. LC and MS conditions were optimized to quantify intact proteins for clinical sample analysis, resulting LOQs between 1 to 5 µg/mL of the mAb concentration in human serum. Through a quick column reproducibility evaluation, reproducible data were generated over two different column lots showing a 0.2-minute shift with less than 20% peak area differences. The cost of this workflow is around $25 in this small-scale study. The estimated cost is about $13 or lower if the IdeS digestion step is bypassed and scaled up to treat a high volume of samples. This modification will generate light and heavy chains of the mAb in the final sample. Cost estimation in an individual laboratory may differ from what is presented in this study due to factors such as laboratory methods, materials used, and system conditions.

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