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Biopharma / Nexera Bio & Q-TOF LCMS-9030

Peptide Mapping of Monoclonal Antibody (mAb) Using Nexera Bio with Q-TOF Mass Spectrometer for Full Sequence Confirmation

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1. Introduction

The manufacturing of antibody drugs has been gaining massive popularity due to their specificity towards target diseases, efficacy and role as potential personalised medicines. Peptide mapping plays a critical role in quality assurance of antibody drugs. It is employed for elucidating of the primary structure of antibody biosimilars. Peptide mapping of enzymatic digest is carried out using a C18 reversed phase column on a inner HPLC or UHPLC [1]. In this report, a biocompatible Nexera Bio UHPLC system is evaluated in terms of repeatability of LC profiling analysis of tryptic digest of human IgG (immunoglobulin G). It is then applied to bevacizumab biosimilar, a recombinant monoclonal IgG1 antibody. Subsequently, LCMS-9030 (Q-TOF) was employed for confirming the protein sequence through accurate mass matching analysis of the peptides detected, which provides comprehensive and reliable structural characterization and sequence information of the biosimilar drug.

2. Experimental

A 10 mg/mL of human IgG (Sigma) and 10 mg/mL of bevacizumab biosimilar were prepared in phosphate-buffered saline and Tris buffer, respectively. Dithiothreitol (DTT) was added to the sample and the mixture was incubated for 30 minutes at 60°C for reducing the protein disulphide bonds. Alkylation of the sample was done by adding iodoacetamide (IAM) followed by incubation at 37°C for 30 minutes in the dark. Trypsin was added and the total volume of the sample was adjusted with ammonium bicarbonate (ABC) buffer to an optimized ratio for subsequent digestion at 37°C for 4 hours. Trifluoroacetic acid (TFA) solution was added and incubated for 5 minutes at room temperature. The obtained sample was centrifuged and the supernatant was collected and injected to Nexera Bio UHPLC and LCMS-9030 for peptide mapping analysis and peptide sequence confirmation, respectively. The analytical conditions on both systems are displayed in **Table 1** and **Table 2**.

Table 1. Analytical conditions of peptide mapping analysis on Shimadzu Nexera Bio UHPLC system

Column	: Shim-pack GISS 5 µm, 250 × 4.6 mm
Mobile phase	: (A) 0.1% trifluoroacetic acid in water (B) 0.1% trifluoroacetic acid in acetonitrile
Flow rate	: 1.0 mL/min
Gradient program	: B Conc. 0% (0 min) → 5% (0 min) → 50% (85 min) → 75% (95 – 100 min) → 0% (103 min) → 0% (115 min)
Column temp.	: 40°C
Injection volume	: 50 µL
Detection	: PDA (210 nm, ref: 360 nm)

Table 2. Analytical conditions of peptide mapping analysis on LCMS-9030 (Q-TOF)

Column	: Shim-pack GISS C18, 5 µm, 250 × 4.6 mm
Mobile phase	: (A) 0.1% trifluoroacetic acid in water (B) 0.1% trifluoroacetic acid in acetonitrile
Flow rate	: 1.0 mL/min
Gradient program	: B Conc. 0% (0 min) → 5% (0 min) → 50% (85 min) → 75% (95 – 100 min) → 0% (103 min) → 0% (115 min)
Column temp.	: 40°C
Injection volume	: 50 µL
Interface	: Heated ESI (positive mode)
MS Mode	: MS scan
TOF mass range	: 250 – 2500 (m/z)
Heat block temp.	: 400°C
DL temp.	: 250°C
Interface temp.	: 300°C
Nebulizing gas	: N2, 3 L/min
Drying gas	: N2, 10 L/min
Heating gas	: Zero air, 10L/min

3. Results and discussion

3.1 Peptide mapping of Human IgG and bevacizumab biosimilar on Nexera Bio UHPLC

Peptide mapping of antibody drugs like biosimilars is one of important characterization analyses regarding the similarity and modification in their primary structures as comparing to the reference product [2]. The HPLC system and method for measuring UV chromatographic profile of tryptic digest antibody must be highly reproducible and robust with well separation resolution and high peak capacity. A Nexera Bio system was adopted in this study, which is a modified Nexera UHPLC system with using carbon-coated pump

head, gold-plated ferrule, stainless steel clad PEEK tubing and ceramic injection needle. A Shim-pack GISS C18 column was employed. The column can sustain extreme acidic condition (pH 1) and thus 0.1% trifluoroacetic acid in water and acetonitrile were used as mobile phases. To facilitate elution of the peptides of tryptic digests, a shallow gradient program of 115 minutes was adopted.

Figures 1 and 2 show the selected chromatograms of tryptic digests of IgG and bevacizumab biosimilar obtained from intra-day and inter-day experiments. The repeatability of RT for IgG was compiled in **Tables 3** and **4**. Six selected peaks (A to F) were used for calculating the RSD. The intra-day repeatability was determined based on the analyses of six continuous injections of same digest. For a period of three days, measurement was repeated to obtain inter-day repeatability with three different tryptic digests. The results show high RT repeatability (RSD<0.5%), confirming the robustness of peptide mapping by using Nexera Bio system.

Table 3 Intra-day repeatability of retention time of IgG tryptic digests (n=6), 2nd day

Peak	Average (min)	Std. Dev. (min)	%RSD (%)
Peak A	5.638	0.006	0.107
Peak B	16.102	0.044	0.270
Peak C	31.023	0.010	0.034
Peak D	50.304	0.008	0.016
Peak E	57.156	0.011	0.020
Peak F	63.722	0.016	0.024

Table 4 Inter-day repeatability of retention time (n = 3 day x 6 injections) of IgG tryptic digests

Peak	Average (min)	Std. Dev. (min)	%RSD (%)
Peak A	5.618	0.025	0.438
Peak B	16.024	0.077	0.482
Peak C	31.001	0.045	0.146
Peak D	50.318	0.064	0.127
Peak E	57.154	0.036	0.063
Peak F	63.744	0.071	0.111

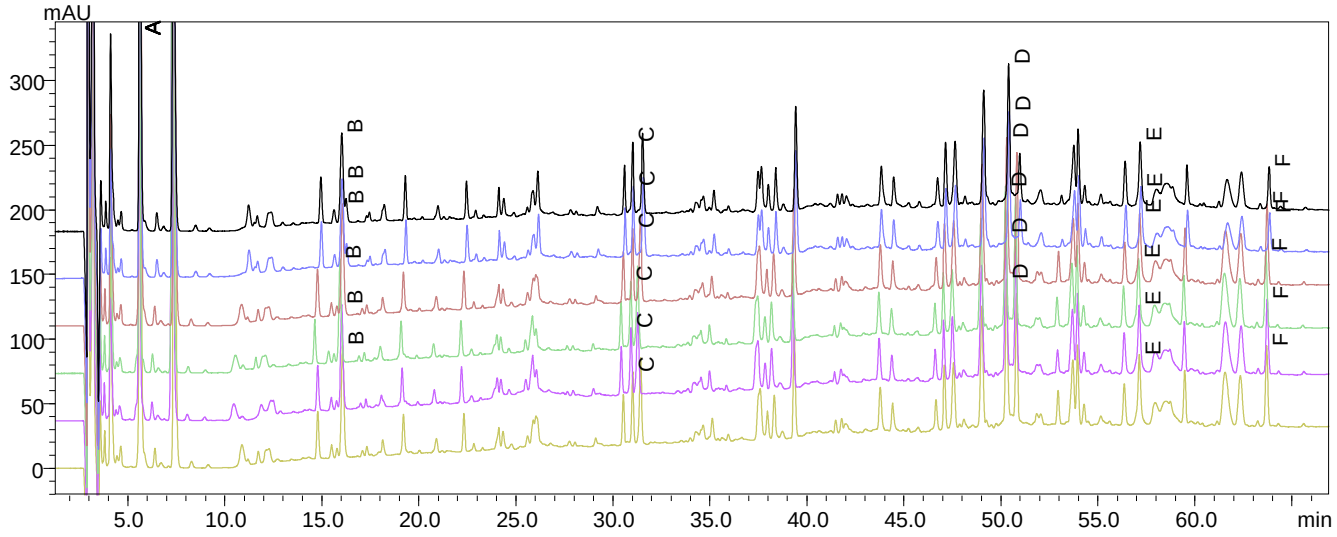

Figure 1. Inter-day repeatability tests of IgG tryptic digest for three days (each subsequent two data represents duplicate injections in the same day) on Nexera Bio UHPLC system

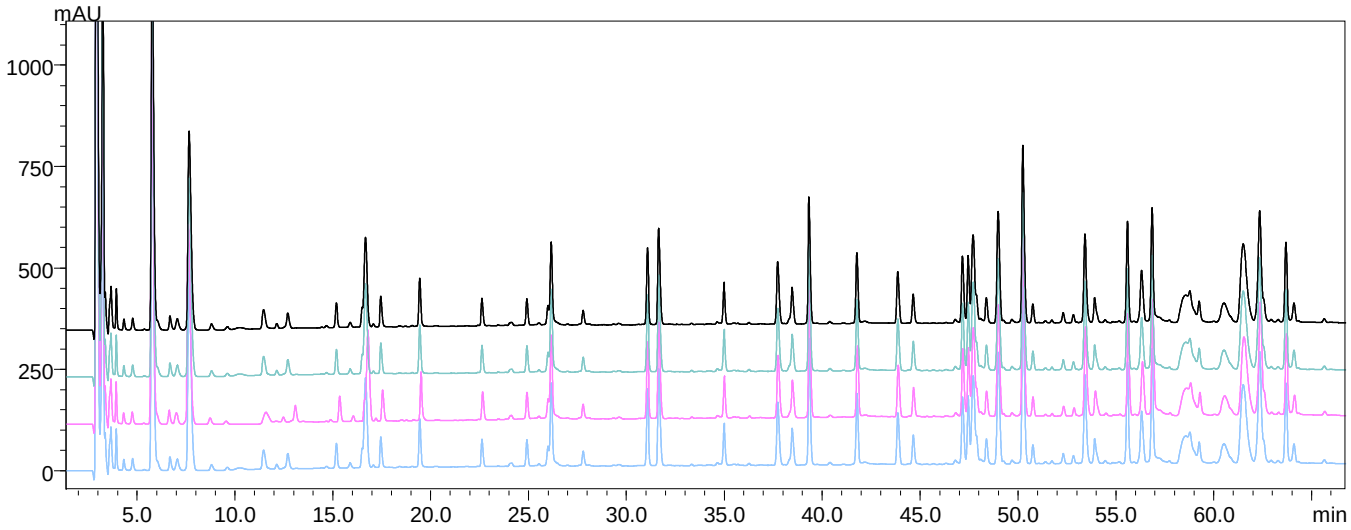

Figure 2. Inter-day repeatability tests of bevacizumab biosimilar tryptic digest for two days (each subsequent two data represents duplicate injection in the same day) on Nexera Bio UHPLC system

3.2 Confirmation of peptides by Q-TOF

The tryptic digest of bevacizumab biosimilar was analysed on LCMS-9030 for confirmation of the peptide sequences based on accurate mass matching. The LC conditions and column used are exactly same as that used on Nexera Bio (Figure 2). As shown in Figure 3A, an almost identical LC profile was obtained with PDA detection (210 nm). More peaks were detected in full range TIC (m/z 240~2500) by Q-TOF as shown in Figures 3B (display only 240~950). A careful data analysis reveals that all of the peptides of bevacizumab tryptic digest in silico [2] were detected based on accurate mass matching including two large peptides, HC40 [152, 215] from heavy chain and LC18 [61, 102] from light chain with their masses of 6713.3145+ and 4660.1337+, respectively. The peptides listed in **Table 5** are confirmed with high reliability by matching the spectrum within 2 ppm mass accuracy and corresponding charge number. For example, as illustrated in **Figure 4**, for peptides HC20 and HC21, their masses match precisely with the sequences of SLSLSPG [445, 451] and ALPAPIEK [332,339] with a mass error of -1.1 ppm and +0.6 ppm, respectively. SLSLSPG is known to be the C-terminal peptide of bevacizumab heavy chain after lysine truncation [3]. Moreover, for peptide LC11 from light chain [190, 206], its both doubly- and triply-charged ions were observed and their masses matched perfectly with theoretical values (error, 0.21 ppm and 2.9 ppm, respectively). The above examples indicate the high reliability of the accurate mass matching for confirmation of the peptides, which confirms the amino acid sequence indirectly. The sequence coverage of the analysis has achieved 100% (excluding the slipped lysine in C-terminal peptide of LC).

The % RSD of RT of the detected peptides acquired (*n*=3) are at 0.1%. This results indicate viability of LCMS-9030 for peptide mapping, which signifies the advantages of Q-TOF in sequence and structural characterization of biosimilars.

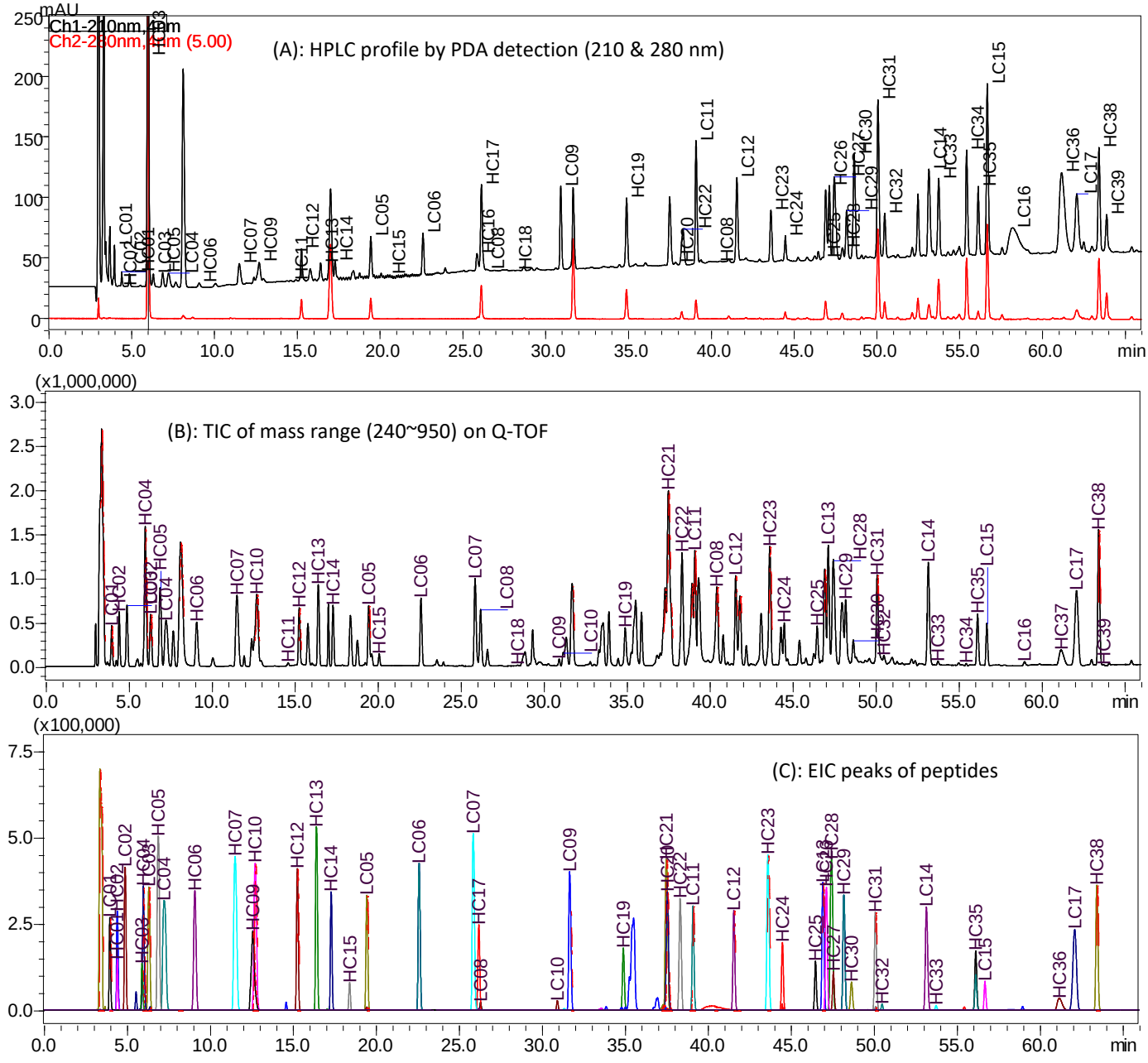

Figure 3. Chromatograms of bevacizumab biosimilar tryptic digest on LCMS-9030. (A) PDA detection (210 nm and 280 nm); (B) TIC of TOF detection and (C) EICs of peptides, as indicated in Table 5.

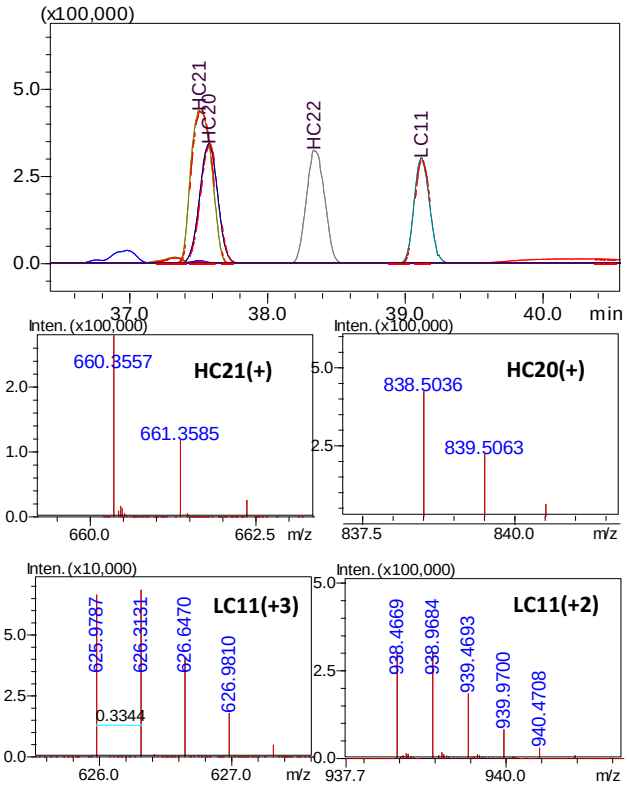

Figure 4. Display of four peptide peaks (top) and their mass spectra (bottom) obtained on Q-TOF LCMS-9030.

4. Conclusions

The Nexera Bio, a biocompatible UHPLC system, with using a Shim-Pack GISS C18 column of 5 µm particle size was proven to be robust and reliable for peptide mapping analysis of tryptic digests of human IgG and a bevacizumab biosimilar product. The results of intra- and inter-day repeatability tests in retention time are satisfactory. The peptide analysis performed with Q-TOF mass spectrometer provides in-depth information in identification of peptides, sequences and modifications. A 100% sequence coverage was achieved for the bevacizumab biosimilar sample studied, which enhances greatly the reliability of peptide mapping. The demonstrated performance of both Nexera Bio and Q-TOF LCMS-9030 signifies their practicability for primary structural characterization of monoclonal antibody and biosimilars in biopharmaceutical research and manufacture.

References

1. Shimadzu Corporation, "Peptide Mapping of Antibody Drugs by Nexera-i", Application News, No. L488, 2015
2. US FDA, "Quality Considerations in Demonstrating Biosimilarity of a Therapeutic Protein Product to Reference Product", Guidance for Industry, 2015.
3. https://skyline.ms/wiki/home/software/Skyline/page.view?name=default

Table 5. Full sequence (100%) confirmation of bevacizumab biosimilar by accurate mass matching of tryptic peptides of on LCMS-9030

Location & Peak No.		RT (min)	Peptide [AA numbers]	m/z & z (mea.)
Heavy chain (HC)	HC26	47.2	EVQLVESGGGLVQPGGSLR (N-Terminal) [0, 18]	941.5054++
	HC34	55.5	LSCAASGYTFTNYGMNWVR [19, 37]	1099.4945++
	HC10	12.7	QAPGK [38, 42]	500.2817+
	HC39	63.9	GLEWVGWINTYTGEPTYAADFK [43, 64]	840.0665+++
	HC03	5.9	RR [65, 66] (missed 1)	331.2185+
	HC27	47.6	RFTFSLDTSK [66, 75] (missed 1)	1201.6238+
	HC29	48.2	FTFSLDTSK [67, 75]	1045.5220+
	HC24	44.5	STAYLQMNSLR [76, 86]	642.3231++
	HC19	34.9	AEDTAVYYCAK [87, 97]	1290.5697+
	HC37	61.3	YPHYYGSSHWYFDVWGQGTLVTVSSASTK [98, 126]	1108.5214+++
	HC28	47.7	GPSVFPLAPSSK [127, 138]	593.8261++
	HC23	43.6	K.STSGGTAALGCLVK.D [139, 152]	661.3420++
	HC40	62.6	DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSV VTVPSSSLGTQTYICNVNHKPSNTK [153, 215]	2238.4435+++
	HC06	9.1	VDK [216, 218]	361.2065+
	HC14	17.4	KVEPK [219, 223] (missed 1)	600.3707+
	HC04	6.0	SCDK [224, 227]	509.2015+
	HC36	61.2	THTCPPCPAPELLGGPSVFLFPPKPK [228, 253]	948.8229+++
	HC22	38.3	DTLMISR [254, 260]	835.4343+
	HC30	48.7	TPEVTCVVVDVSHEDPEVK [261, 279]	1070.0185++
	HC31	50.1	FNWYVDGVEVHNAK [280, 293]	839.4039++
	HC07	11.5	TKPR [294, 297]	501.3136+
	HC18	28.1	EEQYNSTYR [298, 306]	595.2584++
	HC38	63.5	VVSVLTVLHQDWLNGK [307, 322]	904.5067++
	HC12	15.3	EYK [323, 325]	439.2175+
	HC02	4.4	CK [326, 327]	307.1422+
	HC05	6.9	VSNK [328, 331]	447.2554+
	HC21	37.8	ALPAPIEK [332, 339]	838.5038+
	HC13	16.4	TISK [340, 343]	448.2757+
	HC11	14.6	AKGQPR [344, 349] (missed 1)	656.3827+
	HC09	12.6	GQPR [346, 349]	457.2503+
	HC08	40.3	EPQVYTLPPSR [350, 360]	1286.6769+
	HC15	18.4	EEMTK [361, 365]	637.2852+
	HC25	46.6	NQVSLTCLVK [366, 375]	1161.6322+
	HC33	53.7	GFYPSDIAVEWESNGQPENNYK [376, 397]	848.714+++
	HC35	56.2	TTPPVLDSDGSFFLYSK [398, 414]	937.4641++
	HC17	26.2	LTVDK [415, 419]	575.3388+
	HC16	25.9	LTVDKSR [415, 421] (missed 1)	818.4746+
	HC32	50.5	WQQGNVFSCSVMHEALHNHYTQK [422, 444]	934.4261+++
	HC20	37.7	SLSLSPG (C-terminal) [445, 451]	660.3556+
Light Chain (LC)	LC12	41.6	DIQMTQSPSSLSASVGDR (N-terminal) [0, 17]	939.9466++
	LC15	56.7	VTITCSASQDISNYLNWYQQKPGK [18, 41]	934.4561+++
	LC04	7.3	APK [42, 44]	315.2016+
	LC14	53.1	VLIYFTSSLHSGVPSR [45, 60]	881.9776++
	LC18	65.2	FSGSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQ GTK [61, 102]	2302.0501++
	LC07	25.9	VEIK [103, 106]	488.3071+
	LC08	26.3	VEIKR [103, 107] (missed 1)	644.4079+
	LC16	58.3	TVAAPSVFIFPPSDEQLK [108, 125]	649.3452+++
	LC17	62.1	SGTASVVCLLNNFYPR [126, 141]	899.4505++
	LC02	4.9	EAK [142, 144]	347.1915+
	LC09	31.1	VQWK [145, 148]	560.3180+
	LC10	31.1	VDNALQSGNSQESVTEQDSK [149, 168]	712.6598+++
	LC13	47.2	DSTYSLSSTLTLSK [169, 182]	751.8822++
	LC05	19.4	ADYEK [183, 187]	625.2819+
	LC01	4.0	HK [188, 189]	284.1706+
	LC11	39.1	VYACEVTHQGLSSPVTK [190, 206]	938.4669++
	LC06	22.6	SFNR [207, 210]	523.2614+
	LC03	6.3	GEC [211, 213] C-terminal	365.1109+

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