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Application News

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Biopharma / LCMS-9030 (Q-TOF)

In-depth Peptide Mapping of Monoclonal Antibody (mAb) by A *de novo* Peptide Sequencing Method on Q-TOF Mass Spectrometer with Data-independent Acquisition

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❑ Introduction

Peptide mapping is an important and efficient technique for the primary structure characterization of monoclonal antibodies (mAbs). It involves enzymatic digestion (e.g., trypsin) and enables the direct detection of single amino acid changes. Also, it can provide additional information of post-translational modifications (PTMs). However, we have to note that peptide mapping is usually performed in a comparative manner, in which the peptide map of a mAb product is compared to that of a reference in a side-by-side experiment, which is highly dependent on the reproducible sample preparation and extremely time consuming. An in-depth peptide mapping workflow with a *de novo* sequencing method is thus required to verify primary structure of mAb and identify any alterations in structure. An emerging technique, and the focus of this report, is data-independent acquisition (DIA) mode on Q-TOF mass spectrometry. DIA enables a genetic, non-biased approach for the MS/MS data acquisition, in which all the ions in each individual MS/MS event are fragmented without precursor ion isolation and all the fragment ions are measured in a mixed spectrum, ideal for peptide sequencing. In this report, we demonstrated an integrated MS full-scan and MS/MS DIA approach on Shimadzu LCMS™-9030 (Q-TOF) mass spectrometer for *de novo* peptide sequencing of mAb (**Figure 1**).

❑ Experimental

A 5 mg/mL of bevacizumab biosimilar sample solution was prepared in 50 mmol/L Tris-HCl (pH 8.0) buffer. A 20 µL aliquot of the sample was diluted with 80 µL of ammonium bicarbonate (ABC) solution (50 mM), then mixed with 10 µL ProteaseMAX™ (0.5%, w/w) and 10 µL Dithiothreitol (DTT, 0.2 M), incubated at 60°C for 60 min to denature and reduce disulfide bonds. Alkylation was done by adding 30 µL iodoacetamide (IAM, 0.2 M) followed by incubation at 37°C for 60 min in the dark. The sample were diluted with 328 µL ABC solution (50 mM) before trypsin digestion. Sequencing grade trypsin was used for digestion at 37°C for overnight. Finally, 2 µL trifluoroacetic acid (TFA) was added to stop activity of trypsin. The sample was centrifuged and supernatant was collected and injected to LCMS-9030 (Q-TOF) for DIA analysis. The analytical conditions are displayed in **Table 1,** and the MS and MS/MS segments is shown in **Table 2**

Table 1. Analytical conditions on LCMS-9030 (Q-TOF)

LC conditions	
LC system:	Shimadzu Nexera™ X2 UHPLC
Column:	Shim-pack™ GISS-HP, 3 µm, 150 × 3.0 mm 40 °C
Column temperature:	
Flow rate:	0.5 mL/min
Mobile phase A:	0.1% FA + 0.01% TFA in water
Mobile phase B:	0.1% FA + 0.01% TFA in acetonitrile
Gradient program:	B Conc. 0% (0-2 min) → 15% (10 min) → 35% (23 min) → 45% (30 min) → 75% (35-40 min) → 0% (40.1-45 min).
Injection volume:	20 µL
MS conditions	
MS system:	Shimadzu LCMS-9030 (QTOF)
Interface:	Heated ESI (+)
Interface voltage:	4.5 kV
Interface temperature:	300 °C
Nebulizing gas:	N2, 3 L/min
Heating gas flow:	Zero air, 10L/min
DL temperature:	250 °C
Drying gas flow:	N2, 10 L/min
Heat block temperature:	400 °C
MS mode:	MS
Mass range:	100 - 2000 m/z
MS/MS mode:	DIA
Mass segment:	From 210 to 1690 by 40 m/z
Collision Energies:	35 ± 17 V

❑ Results and Discussion

A. MS and MS/MS data extraction

The raw MS data file (.lcd) was exported as .mzML data by LabSolutions™ s/w, and then uploaded into MS-DIAL s/w [2] for both MS and MS/MS data extraction. The default parameters in MS-DIAL were applied for peak detection and extraction with the minimum peak height setting at 1500 amplitude. Finally, 11,852 mass peaks including the information of precursor *m/z*, retention time, peak height, adduct ions, and MS/MS fragment ions were extracted (data not shown).

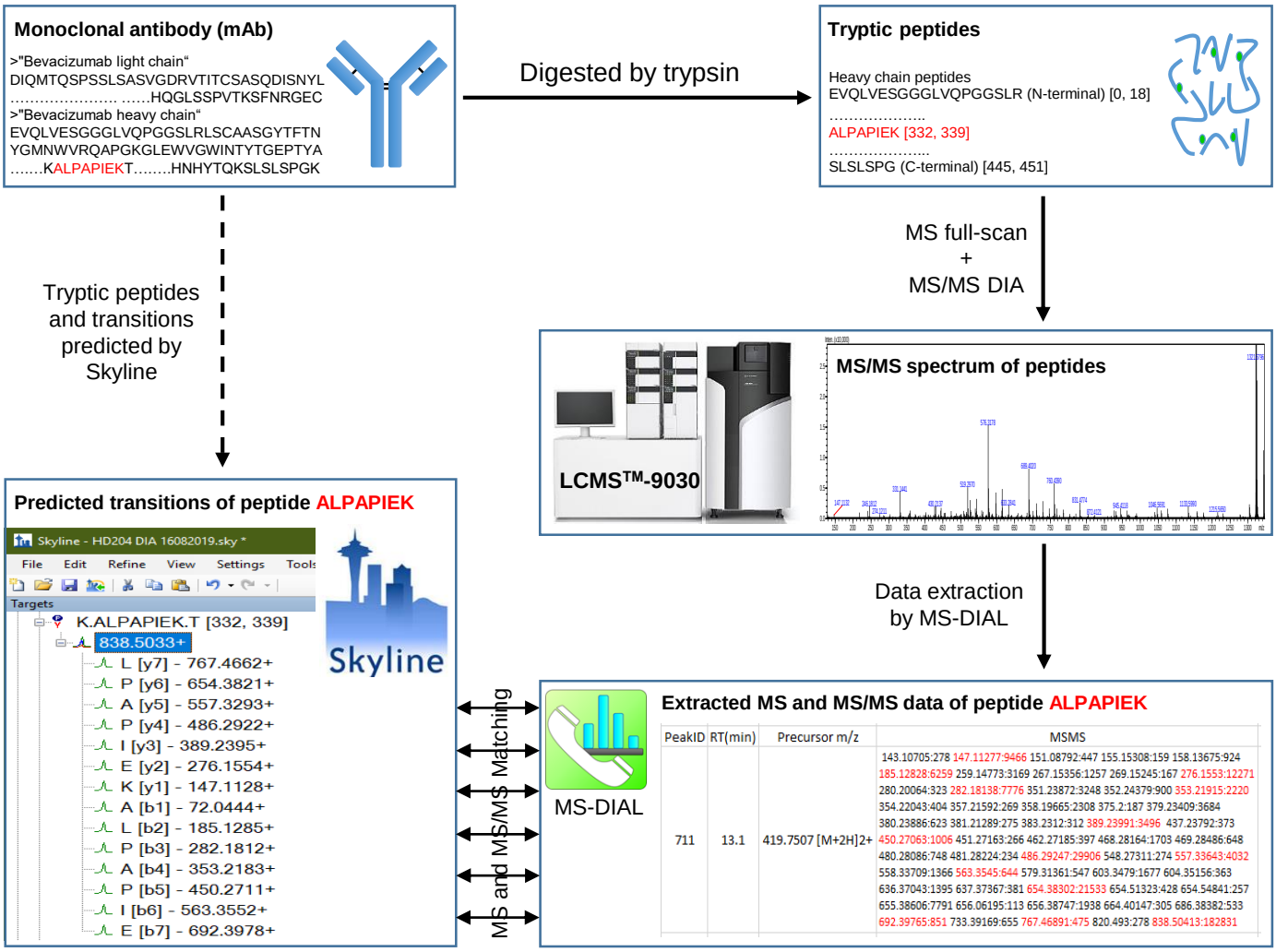

Figure 1. The *de novo* peptide sequencing approach on *LCMS-9030 (Q-TOF) for characterization* of mAb. The peptide ALPAPIEK was used as an example for illustration.

Table 2. MS and MS/MS experiment file

Experiment	MS Type	Min m/z	Max m/z
0	MS	100	2000
1	MS/MS	210	250
2	MS/MS	250	290
3	MS/MS	290	330
4	MS/MS	330	370
5	MS/MS	370	410
6	MS/MS	410	450
7	MS/MS	450	490
8	MS/MS	490	530
9	MS/MS	530	570
10	MS/MS	570	610
11	MS/MS	610	650
12	MS/MS	650	690
.	.	.	.
.	.	.	.
.	.	.	.
30	MS/MS	1370	1410
31	MS/MS	1410	1450
32	MS/MS	1450	1490
33	MS/MS	1490	1530
34	MS/MS	1530	1570
35	MS/MS	1570	1610
36	MS/MS	1610	1650
37	MS/MS	1650	1690

B. Theoretical MS/MS transitions of peptides

The amino acid sequence of bevacizumab downloaded from DrugBank https://www.drugbank.ca was imported into Skyline s/w [3] for MS/MS transitions prediction. In peptide settings, tryptic peptides with carbamidomethyl modification were selected. In transition settings, only singly charged y and b ions (+) were predicted, as they are the most common fragmentations observed in low energy collisions.

C. Peptide sequencing

The main aim of this study is to explore the potential of MS/MS DIA method for in-depth peptide mapping at *de novo* peptide sequencing level. Based on our previous application news of AD-0212B [1], 42 and 19 peptides were measured from the heavy-chain and light-chain respectively, covering 100% amino acid sequence of bevacizumab biosimilar. In the present report, we focus on the same peptides for *de novo* peptide sequencing. By matching the extracted MS/MS spectra data by MS-DIAL to the theoretical MS/MS transitions of tryptic peptide of bevacizumab in Skyline, all the 61 peptides were well identified and verified for their amino acid sequences (**Tables 3 and 4**).

Table 3. *De novo* peptide sequencing of bevacizumab biosimilar heavy chain by MS/MS DIA approach on LCMS-9030

Peak No.	Peptide [AA numbers]	MS (m/z&z, mea.)	RT (min)	MS/MS (m/z, mea.) matched with predicted transitions in Skyline s/w
HC01	-.EVQLVESGGGLVQPGGSLR.L [1, 19]	941.5052++	17.23	1313.6813 (y14); 1184.6338 (y13); 1097.6059 (y12); 1040.5843 (y11); 983.5615 (y10); 926.5446 (y9); 813.4589 (y8); 714.3893 (y7); 586.3310 (y6); 489.2777 (y5); 432.2592 (y4); 375.2331 (y3); 288.2014 (y2); 175.1183 (y1); 229.1159 (b2); 357.1755 (b3); 470.2602 (b4); 569.3301 (b5); 698.3733 (b6); 785.4051 (b7); 842.4365 (b8); 899.4452 (b9); 956.4699 (b10); 1069.5449 (b11); 1168.6183 (b12); 1296.6757 (b13)
HC02	R.LS**C**AASGYTFTNYGMNWVR.Q [20, 38]	1099.4940++	20.67	1608.7324 (y13); 1287.5968 (y10); 1140.5254 (y9); 925.4361 (y7); 762.3740 (y6); 574.3081 (y4); 274.1845 (y2); 175.1187 (y1); 647.2755 (b7); 1493.6380 (b14)
HC03	R.QAPGK.G [39, 43]	500.2829+	3.50	204.1326 (y2)
HC04	K.GLEWVGWINTYTGEPTYAADFK.R [44, 65]	840.0690+++	24.13	1690.7969 (y15); 1577.7154 (y14); 1463.6656 (y13); 1362.6230 (y12); 1199.5587 (y11); 1098.5108 (y10); 1041.4901 (y9); 912.4464 (y8); 815.3941 (y7); 714.3451 (y6); 551.2815 (y5); 480.2456 (y4); 409.2080 (y3); 294.1815 (y2); 147.1125 (y1); 171.1124 (b2); 300.1548 (b3); 486.2344 (b4); 585.3038 (b5); 642.3237 (b6); 828.4038 (b7); 941.4867 (b8); 1055.5297 (b9); 1156.5748 (b10); 1319.6389 (b11); 1420.6792 (b12); 1477.7128 (b13); 1606.7544 (b14)
HC38	K.GLEWVGWINTYTGEPTYAADFKR.R [44, 66] (missed 1)	892.1030+++	23.00	1355.6526 (y12); 1068.5361 (y9); 450.2716 (y3); 171.1125 (b2); 300.1551 (b3); 642.3293 (b7); 941.4961 (b8)
HC39	K.RR.F [66, 67] (missed 1)	331.2198+	2.03	175.1195 (y1)
HC40	R.RFTFSLDTSK.S [67, 76] (missed 1)	601.3149++	16.40	1045.5218 (y9); 898.4441 (y8); 797.4053 (y7); 650.3378 (y6); 563.3060 (y5); 450.2189 (y4); 335.1922 (y3); 234.1444 (y2); 147.1124 (y1); 157.1072 (b1); 304.1762 (b2); 405.2223 (b3); 552.2912 (b4); 639.3256 (b5); 752.4083 (b6); 867.4357 (b7); 968.4825 (b8); 1055.5141 (b9)
HC05	R.FTFSLDTSK.S [68, 76]	523.2642++	17.40	898.4521 (y8); 797.4041 (y7); 650.3358 (y6); 563.3035 (y5); 450.2190 (y4); 335.1921 (y3); 234.1446 (y2); 147.1126 (y1); 249.1231 (b2); 396.1918 (b3); 483.2211 (b4); 596.3092 (b5); 711.3365 (b6)
HC06	K.STAYLQMNSLR.A [77, 87]	642.3186++	15.97	1095.5611 (y9); 1024.5251 (y8); 861.4611 (y7); 748.3765 (y6); 620.3179 (y5); 489.2774 (y4); 375.2346 (y3); 288.2020 (y2); 175.1186 (y1); 189.0862 (b2); 260.1238 (b3); 423.1864 (b4); 536.2715(b5); 664.3289 (b6); 795.3706 (b7); 909.4114 (b8); 996.4463 (b9); 1109.5303 (b10)
HC07	R.AEDTAVYY**C**AK.Y [88, 98]	645.7824++	12.43	1219.5344 (y10); 1090.4877 (y9); 975.4592 (y8); 874.4123 (y7); 803.3761 (y6); 704.3072 (y5); 541.2430 (y4); 378.1801 (y3); 218.1495 (y2); 147.1121 (y1); 201.0865 (b2); 316.1142 (b3); 417.1609 (b4); 488.1986 (b5); 587.2665 (b6); 750.3321 (b7); 913.3932 (b8); 1073.4188 (b9); 1144.4664 (b10)
HC08	K.YPHYYGSSHWYFDVWGQGTLVTVSSASTK.G [99, 127]	1108.5200+++	22.00	1521.7817 (y15); 1150.6364 (y12); 992.5611 (y10); 879.4779 (y9); 780.4088 (y8); 679.3571 (y7); 580.2946 (y6); 493.2662 (y5); 335.1915 (y3); 147.1111 (y1); 261.1220 (b2); 398.1855 (b3); 561.2379 (b4); 1802.7533 (b14)
HC09	K.GPSVFPLAPSSK.S [128, 139]	593.8290++	17.10	1032.5725 (y10); 945.5395 (y9); 846.4723 (y8); 699.4035 (y7); 602.3510 (y6); 489.2650 (y5); 418.2294 (y4); 321.1758 (y3); 234.1445 (y2); 147.1128 (y1); 155.0812 (b2); 242.1131 (b3); 341.1816 (b4); 488.2507 (b5); 698.3879 (b7); 769.4247 (b8); 953.5011 (b10); 1040.5395 (b11)
HC10	K.STSGGTAALG**C**LVK.D [140, 153]	661.3535++	15.60	1133.6011 (y12); 1046.5643 (y11); 989.5407 (y10); 831.4741 (y8); 760.4365 (y7); 689.3999 (y6); 576.3168 (y5); 519.2965 (y4); 359.2633 (y3); 246.1812 (y2); 147.1122 (y1); 189.0866 (b2); 276.1187 (b3); 333.1410 (b4); 390.1581 (b5); 803.3955 (b10)
HC11	K.DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYI**C**NVNHKPSNTK.V [154, 216]	1679.0850++++	24.97	2433.1811 (y22); 1675.8302 (y14;); 1298.6312 (y11); 925.4857 (y8); 811.4421 (y7); 674.3846 (y6); 546.2880 (y5); 449.2345 (y4); 362.2010 (y3); 279.0973 (b2); 426.1657 (b3); 523.2169 (b4); 652.2640 (b5); 749.3157 (b6); 848.3809 (b7); 949.4295 (b8); 1048.5006 (b9); 1135.5261 (b10); 1321.6154 (b11)
HC12	K.VDK.K [217, 219]	361.2089+	2.53	262.1394 (y2); 147.1124 (y1); 215.1020 (b2)
HC41	K.VDKK.V [217, 220] (missed 1)	489.3030+	2.13	390.2335 (y3); 275.2073 (y2); 147.1124 (y1); 215.1014 (b2); 343.1984 (b3)
HC42	K.KVEPK.S [220, 224] (missed 1)	600.3716+	4.50	472.2757 (y4); 373.2072 (y3); 244.1648 (y2); 147.1128 (y1); 129.1020 (b1); 228.1692 (b2); 357.2127 (b3); 454.2650 (b4)
HC13	K.VEPK.S [221, 224]	472.2766+	5.20	373.2067 (y3); 244.1651 (y2); 147.1124 (y1); 229.1180 (b2)
HC14	K.S**C**DK.T [225, 228]	509.2029+	2.20	422.1701 (y3); 262.1393 (y2); 147.1121 (y1); 248.0697 (b2); 363.0964 (b3)
HC15	K.THT**C**PPCPAPELLGGPSVFLFPPKPK.D [229, 254]	948.8240+++	21.93	1991.1378 (y19); 1823.0460 (y17); 1596.9382 (y15); 1483.8667 (y14); 1370.7831 (y13); 1313.7612 (y12); 1256.7411 (y11); 1159.6828 (y10); 1072.6526 (y9); 973.5832 (y8); 826.5185 (y7); 713.4339 (y6); 566.3662 (y5); 469.3120 (y4); 372.2594 (y3); 244.1654 (y2); 147.1121 (y1); 239.1134 (b2); 340.1620 (b3); 500.1916 (b4); 597.2481 (b5); 694.3050 (b6); 854.3287 (b7); 951.3388 (b8); 1022.4183 (b9); 1248.5186 (b11); 1361.5935 (b12); 1474.6891 (b13); 1531.6999 (b14); 1588.72011 (b15)
HC16	K.DTLMISR.T [255, 261]	418.2220++	13.27	506.2759 (y4); 375.2371 (y3); 262.1507 (y2); 175.1185 (y1); 217.0821 (b2); 330.1649 (b3)
HC17	R.TPEVT**C**VVVDVSHEDPEVK.F [262, 280]	713.6810+++	17.27	1451.7383 (y13); 1352.6719 (y12); 1253.6011 (y11); 1154.5336 (y10); 1039.5043 (y9); 940.4366 (y8); 853.4050 (y7); 716.3464 (y6); 587.3035 (y5); 472.2759 (y4); 375.2241 (y3); 246.1809 (y2); 147.1123 (y1); 102.0548 (b1); 199.1074 (b2); 328.1496 (b3); 427.2175 (b4); 528.2655 (b5); 688.3004 (b6); 886.4346 (b8); 985.5012 (b9); 1100.5266 (b10); 1199.6048 (b11)); 1286.6326 (b12)
HC18	K.FNWYVDGVEVHNAK.T [281, 294]	839.3967++	17.67	1416.6915 (y12); 1230.6108 (y11); 1067.5472 (y10); 968.4800 (y9); 853.4513 (y8); 796.4317 (y7); 697.3622 (y6); 568.3196 (y5); 469.2512 (y4); 332.1928 (y3); 218.1494 (y2); 147.1122 (y1); 262.1182 (b2); 448.1976 (b3); 611.2613 (b4); 710.3315 (b5); 825.3561 (b6); 882.3832 (b7); 981.4466 (b8); 1110.4916 (b9); 1209.5591 (b10); 1346.6165 (b11); 1460.6575 (b12); 1531.7027 (b13)
HC19	K.TKPR.E [295, 298]	501.3149+	2.40	175.1183 (y1); 230.1512 (b2)
HC20	R.EEQYNSTYR.V [299, 307]	595.2588++	10.20	803.3704 (y6); 640.3070 (y5); 526.2662 (y4); 439.2217 (y3)
HC21	R.VVSVLTVLHQDWLNGK.E [308, 323]	904.5058++	22.96	1609.8697 (y14); 1522.8416 (y13); 1423.7689 (y12); 1310.6867 (y11); 1209.6407 (y10); 1110.5688 (y9); 997.4862 (y8); 860.4268 (y7); 732.3675 (y6); 617.3403 (y5); 431.2606 (y4); 318.1767 (y3); 204.1340 (y2); 147.1122 (y1); 199.1439 (b2); 286.1754 (b3); 385.2453 (b4); 498.3291 (b5); 599.3741 (b6); 698.4438 (b7); 811.5251 (b8); 948.5889 (b9); 1076.6439 (b10); 1191.6743 (b11); 1377.7482 (b12); 1490.8335 (b13); 1604.8843 (b14); 1661.8978 (b15)
HC22	K.EYK.C [324, 326]	439.2183+	5.67	310.1709 (y2); 147.1132 (y1)
HC23	K.**C**K.V [327, 328]	307.1427+	2.03	147.1122 (y1); 161.0369 (b1)
HC24	K.VSNK.A [329, 332]	447.2567+	2.30	348.1872 (y3); 261.1552 (y2); 147.1130 (y1); 187.1073 (b2); 301.1498 (b3)
HC25	K.ALPAPIEK.T [333, 340]	419.7507++	13.07	767.4689 (y7); 654.3830 (y6); 557.3364 (y5); 486.2925 (y4); 389.2399 (y3); 276.1553 (y2); 147.1128 (y1); 185.1283 (b2); 282.1814 (b3); 353.2192 (b4); 450.2706 (b5); 563.3545 (b6); 692.3977 (b7)
HC26	K.TISK.A [341, 344]	448.2760+	5.60	234.1439 (y2); 147.1137 (y1); 215.1399 (b2); 302.1733 (b3)
HC27	K.AK.G [345, 346]	218.1499+	2.03	147.1129 (y1)
HC28	K.GQPR.E [347, 350]	457.2515+	3.43	272.1720 (y2); 175.1181 (y1); 186.0868 (b2)
HC29	R.EPQVYTLPPSR.E [351, 361]	643.8400++	14.33	833.4492 (y7); 670.3902 (y6); 569.3416 (y5); 456.2560 (y4); 359.2071 (y3); 175.1182 (y1); 355.1563 (b3)
HC30	R.EEMTK.N [362, 366]	637.2858+	6.67	379.2018 (y3); 248.1603 (y2); 147.1121 (y1); 259.0912 (b2); 390.1361 (b3)
HC31	K.NQVSLT**C**LVK.G [367, 376]	581.3180++	16.73	243.1090 (b1); 342.1818 (b3)
HC32	K.GFYPSDIAVEWESNGQPENNYK.T [377, 398]	848.7148+++	20.10	1693.7510 (y14); 1594.6710 (y13); 1465.6309 (y12); 1279.5533 (y11); 1150.5127 (y10); 1063.4797 (y9); 949.4381 (y8); 892.4161 (y7); 764.3576 (y6); 667.2997 (y5); 538.2611 (y4); 424.2201 (y3); 310.1755 (y2); 147.1119 (y1); 205.0973 (b2); 368.1611 (b3); 465.2116 (b4); 667.221 (b5); 780.3547 (b7); 851.3947 (b8); 950.4615 (b9); 1079.5090 (b10); 1265.5870 (b11); 1394.6275 (b12); 1595.6536 (b13); 1780.7855 (b14)
HC33	K.TTPPVLDSDGSFFLYSK.L [399, 415]	625.3116+++	20.93	1063.5161 (y9); 948.4813 (y8); 891.4625 (y7); 804.4308 (y6); 657.3602 (y5); 510.2934 (y4); 397.2081 (y3); 234.1449 (y2); 147.1125 (y1); 203.1032 (b2); 397.2081 (b4); 496.2781 (b5); 609.3581 (b6); 724.3839 (b7); 938.4703 (b10); 1070.5037 (b11); 1217.5710 (b12)
HC34	K.LTVDK.S [416, 420]	575.3400+	9.03	262.1395 (y2); 147.1128 (y1); 215.1385 (b2)
HC35	K.SR.W [421, 422]	262.1506+	2.03	175.1183 (y1)
HC36	R.WQQGNVFS**C**SVMHEALHNHYTQK.S [423, 445]	934.4266+++	17.13	376.2277 (y3); 147.1130 (y1); 315.1444 (b2)
HC37	K.SLSLSPG.- [446, 452] (C-terminal)	660.3680+	13.80	260.1249 (y3); 173.0908 (y2); 201.1224 (b2); 288.1556 (b3); 488.2747 (b5)

Table 4. *De novo* peptide sequencing of bevacizumab biosimilar light chain by MS/MS DIA approach on LCMS-9030

Peak No.	Peptide [AA numbers]	MS (m/z&z, mea.)	RT (min)	MS/MS (m/z, mea.) matched with predicted transitions in Skyline s/w
LC01	-.DIQMTQSPSSLSASVGDR.V [1, 18]	939.9467++	15.13	1391.6845 (y14); 1162.5622 (y12); 1075.5380 (y11); 978.4829 (y10); 691.3380 (y7); 604.2981 (y6); 533.2642 (y5); 446.2338 (y4); 347.1694 (y3); 175.1192 (y1); 357.1749 (b3); 1075.5380 (b10)
LC02	R.VTIT**C**SASQDISNYLNWYQQKPGK.A [19, 42]	934.4571+++	20.83	557.3413 (y5); 301.1873 (y3) ; 201.1217 (b2); 948.4503 (b9)
LC03	K.APK.V [43, 45]	315.2036+	2.30	244.1651 (y2); 147.1123 (y1); 169.0970 (b2)
LC04	K.VLIYFTSSLHSGVPSR.F [46, 61]	881.9708++	18.83	1437.7128 (y13); 1274.6524 (y12); 1127.5804 (y11); 1026.5336 (y10); 939.5010 (y9); 852.4689 (y8); 739.3851 (y7); 602.3267 (y6); 515.2926 (y5); 458.2716 (y4); 359.2037 (y3); 262.1487 (y2); 175.1187 (y1); 213.1594 (b2); 326.2435 (b3); 489.3059 (b4); 636.3748 (b5); 737.4263 (b6); 911.4881 (b8); 1024.5651 (b9); 1161.6352 (b10); 1248.6674 (b11); 1305.6857 (b12); 1404.7547 (b13)
LC05	R.FSGSGSGTDFTLTISSLQPEDFATYY**C**QQYSTVPWTFGQGTK.V [62, 103]	1554.0430+++	25.23	2214.0026 (y18); 2050.9346 (y17); 1887.8758 (y16); 1727.8412 (y15); 1599.7832 (y14); 1471.7274 (y12); 1308.6560 (y12); 1221.6252 (y11); 1120.5782 (y10); 1021.5104 (y9); 924.4617 (y8); 738.3787 (y7); 637.3314 (y6); 490.2612 (y5); 433.2402 (y4); 305.1811 (y3); 248.1590 (y2); 147.1137 (y1); 436.1843 (b5); 523.2132 (b6); 681.2908 (b8); 796.3093 (b9); 943.3770 (b10); 1044.4258 (b11); 1157.5080 (b12); 1259.5585 (b13); 1371.6421 (b14); 1458.6767 (b15); 1545.7069 (b16); 1658.7929 (b17); 1786.8490 (b18); 2346.0809 (b23)
LC06	K.VEIK.R [104, 107]	488.3092+	8.77	389.2385 (y3); 260.1968 (y2); 147.1125 (y1); 229.1181 (b2); 342.2025 (b3)
LC18	K.VEIKR.T [104, 108] (missed 1)	644.4094+	8.47	545.3455 (y4); 416.2978 (y3); 303.2140 (y2); 175.1187 (y1); 229.1182 (b2); 342.2010 (b3); 470.3028 (b4)
LC19	K.RTVAAPSVFIFPPSDEQLK.S [108, 126] (missed 1)	1051.5680++	20.50	913.4683 (y8)
LC07	R.TVAAPSVFIFPPSDEQLK.S [109, 126]	973.5168++	21.70	1745.9188 (y16); 1674.8718 (y15); 1603.8408 (y14); 1506.7970 (y13); 1419.7409 (y12); 1320.6803 (y11); 1173.6172 (y10); 1060.5208 (y9); 913.4617 (y8); 816.4173 (y7); 632.3204 (y5); 517.2991 (y4); 272.1599 (b3); 626.3482 (b7); 1033.5766 (b10)
LC08	K.SGTASVV**C**LLNNFYPR.E [127, 142]	899.4515++	23.17	1481.7463 (y12); 1394.7272 (y11); 1295.6600(y10); 1196.5948 (y9); 1036.5639 (y8); 923.4729 (y7); 810.3884 (y6); 696.3476 (y5); 582.3045 (y4); 435.2353 (y3); 272.1711 (y2); 175.1189 (y1)
LC09	R.EAK.V [143, 145]	347.1935+	2.07	218.1494 (y2); 147.1124 (y1); 201.0867 (b2)
LC10	K.VQWK.V [146, 149]	560.3175+	10.77	461.2505 (y3); 333.1920 (y2); 147.1125 (y1); 228.1339 (b2); 414.2133 (b3)
LC11	K.VDNALQSGNSQESVTEQDSK.D [150, 169]	1068.4870++	11.20	1807.8389 (y17); 1736.7939 (y16); 1495.6525 (y14); 1408.6193 (y13); 1351.5945 (y12); 1237.5567 (y11); 1150.5233 (y10); 1022.4644 (y9); 893.4209 (y8); 806.3889 (y7); 707.3203 (y6); 606.2720 (y5); 477.2291 (y4); 349.1714 (y3); 234.1445 (y2); 147.1119 (y1); 215.1024 (b2); 329.1450 (b3); 400.1817 (b4); 513.2674 (b5); 641.3246 (b6); 728.3561 (b7); 785.3742 (b8); 899.4214 (b9); 986.4566 (b10); 1114.5108 (b11); 1243.5513 (b12); 1330.5941 (b13); 1429.6534 (b14); 1530.7038 (b15); 1659.7397 (b16); 1787.8008 (b17); 1902.8346 (b18); 1989.8593 (b19)
LC12	K.DSTYSLSSTLTLSK.A [170, 183]	751.8830++	17.17	1300.7064 (y12); 1199.6517 (y11); 1036.5889 (y10); 949.5564 (y9); 836.4713 (y8); 749.4399 (y7); 662.4086 (y6); 561.3599 (y5); 448.2763 (y4); 347.2284 (y3); 234.1445 (y2); 147.1119 (y1); 203.0654 (b2); 304.1140 (b3); 467.1782 (b4); 554.2092 (b5); 667.2939 (b6); 754.3271 (b7); 841.3568 (b8); 942.4002 (b9); 1055.4876 (b10); 1156.5379 (b11); 1269.6205 (b12); 1356.6489 (b13)
LC13	K.ADYEK.H [184, 188]	625.2830+	7.60	439.2176 (y3); 147.1110 (y1); 350.1302 (b3)
LC14	K.HK.V [189, 190]	284.1717+	2.00	147.1125 (y1); 138.0661 (b1)
LC15	K.VYA**C**EVTHQGLSSPVTK.S [191, 207]	938.4586++	13.43	531.3121 (y5); 444.2809 (y4); 960.4259 (b8)
LC16	K.SFNR.G [208, 211]	523.2572+	7.73	436.2312 (y3); 289.1615 (y2); 175.1188 (y1); 88.0390 (b1); 235.1074 (b2); 349.1499 (b3)
LC17	R.GE**C**.- [212, 214] (C-terminal)	365.1131+	2.53	308.0896 (y2); 179.0484 (y1); 187.0712 (b2)

Here we present peptide ALPAPIEK [332, 339] (HC25) as an example to illustrate the DIA workflow for *de novo* peptide sequencing. After trypsin digestion, the peptide was measured by LCMS-9030 (Q-TOF) both in MS and DIA events. Utilizing the MS-DIAL s/w, the MS data of HC25 including *m/z* (419.7507++) and retention time (13.07 min) and its corresponding MS/MS spectral data (e.g., 147.1128, 185.1283, 276.1553...) were extracted (Refer to the bottom right in **Figure 1**). In Skyline s/w, y and b ion transitions of HC25 were predicted (Refer to the bottom left in **Figure 1**). The extracted MS/MS data was well matched with the predicted transitions, which verify amino acid sequence of peptide ALPAPIEK.

D. Post-translational modifications (PTMs)

In addition to providing primary structure confirmation of mAb, peptide mapping by Q-TOF can determine PTMs, such as functional group addition (e.g., phosphorylation, acylation, glycosylation, etc.) and change in physico-chemical properties of amino acids (e.g., citrullination, deamidation, oxidation, etc.). We didn't pay too much attention to PTMs in the present report, however, some PTMs, such as N-glycosylation of peptide HC20 with G0F (*m/z* 1317.5273++ @ 9.67 min) and c-terminal lysine processing (HC37) were observed .

❑ Conclusions

A simple and straightforward MS/MS DIA approach on Shimadzu LCMS-9030 (Q-TOF) mass spectrometer was demonstrated for in-depth peptide mapping of mAb based on *de novo* peptide sequencing.

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