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A Data-independent Acquisition (DIA) Approach on Q-TOF Mass Spectrometry for In-depth Peptide Mapping of Monoclonal Antibodies

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1. Overview

○ A data-independent acquisition (DIA) approach for *de novo* peptide sequencing was developed on LCMS-9030 Q-TOF mass spectrometer

○ The proposed method can be used to accurately and precisely characterize peptide sequences of monoclonal antibodies (mAbs)

2. Introduction

Peptide mapping plays a critical role in quality assurance of mAbs and is widely applied for primary structure characterization. However, we have to understand that peptide mapping is usually performed in a comparative manner, in which the peptide map of a proposed mAb product is compared to that of a reference product in a side-by-side experiment, which is extremely time consuming and highly dependent on the reproducible sample preparation. An in-depth peptide mapping method is thus required to characterize peptide sequences of mAbs.

An emerging technique, and the focus of this report, is DIA mode on Q-TOF mass spectrometry. DIA enables a genetic, unbiased 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 [1].

In this study, we demonstrated an integrated DIA approach on LCMS-9030 Q-TOF mass spectrometer for *de novo* peptide sequencing of mAbs.

3. Methods

The mAb sample is 1 mg/mL bevacizumab biosimilar solution in 50 mmol/L Tris-HCl buffer (pH 8.0). 100 µL aliquot of the mAb sample was mixed with 10 µL proteaseMAX™ (0.5%, w/w) and 10 µL dithiothreitol (DTT, 0.2 M), incubated at 60ºC for 60 min to denature and reduce disulfide bonds. Then, adding 30 µL iodoacetamide (IAA, 0.2 M) for alkylation by incubation at 37ºC for 60 min in the dark. After dilution with 328 µL of 50 mM ammonium bicarbonate solution, the sample was digested by 20 µL sequencing grade trypsin at 37ºC for overnight. Finally, 2 µL trifluoroacetic acid (TFA) was added to stop activity of trypsin. The obtained sample was centrifuged, and the supernatant was collected for DIA analysis on the Shimadzu LCMS-9030 Q-TOF mass spectrometer, where DIA data acquisition was performed with a 40 Da fixed mass window from 210-1690 m/z acquiring high-resolution MS/MS in each cycle. The analytical conditions are displayed in **Table 1.**

Table 1. Analytical conditions on LCMS-9030

Column	Shim-pack GISS-HP (150 mm x 3.0 mm; 3 µm)
Flow rate	0.5 mL/min
Mobile phase	A: 0.1% FA + 0.01% TFA in water; B: 0.1% FA + 0.01% TFA in acetonitrile
Gradients	B Conc. 0% (0-2 min) → 15% (10 min) → 35% (23 min) → 45% (30 min) → 75% (35-40 min) → 0% (40.1-45 min).
Oven temp.	40ºC
Injection vol.	20 µL
Interface & temp.	Heated ESI (+), 300ºC
Mass range	MS: 100-2000 *m/z*; MS/MS DIA: from 210 to 1690 by 40 *m/z*
Interface voltage	4.5 KV
Heat Block temp.	400ºC
DL temp.	250ºC
Nebulizing gas flow	N_2, 3.0 L/min
Drying gas flow	N_2, 15.0 L/min
Heating gas flow	Zero air, 10.0 L/min

4. Results

4-1. DIA approach for peptide mapping

The objective of this study is to explore the potential of DIA method for in-depth peptide mapping of mAbs at *de novo* sequencing level, as shown in Figure 1.

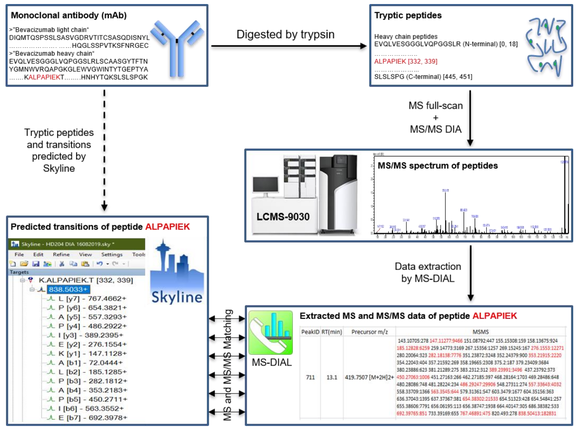

Figure 1. The DIA approach on LCMS-9030 Q-TOF mass spectrometer for *de novo* peptide sequencing of bevacizumab biosimilar (peptide ALPAPIEK was used as an example for illustration).

4-2. 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 data extraction. The default parameters in MS-DIAL were applied for peak detection and extraction with the minimum peak height setting at 1,500 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).

4-3. Theoretical MS/MS transitions of peptides

The amino acid sequence of bevacizumab downloaded from DrugBank https://www.drugbank.ca was imported into the Skyline s/w [3] for MS/MS transitions prediction. In peptide settings, the tryptic peptides with a fixed modification of carboxymethyl (C) 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.

4-4. Peptide sequencing

Based on our previous application news of AD-0212B [4], 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 2 and 3**).

Table 2. *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.LSCAASGYTFTNYGMNWVR.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)
HC39	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.RTFTSLDTSK.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.AEDTAVYYCAK.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.STSGGTAALGCLVK.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); 576.3169 (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); 893.3955 (b10)
HC11	K.DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTK.V [154, 216]	1679.0850++++	24.91	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.V [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.SCDK.T [225, 228]	509.2029+	2.20	422.1701 (y3); 262.1393 (y2); 147.1121 (y1); 248.0697 (b2); 363.0964 (b3)
HC15	K.THTCPPCPAPELLGGPSVFLFPPKPK.D [229, 254]	948.8240+++	21.93	1991.1378 (y19); 1823.0460 (v17); 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.TPEVTCVVVDVSHEDPEVK.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.CK.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.NQVSLTCLVK.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); 496.2791 (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.WQQGNVFSCSVMHEALHNHYTQK.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 3. *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.VTITCSASQDISNYLNWYQQKPGK.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.FSGSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQGTK.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.SGTASVVCLLNNFYPR.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.VYACEVTHQGLSSPVTK.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.GEC.- [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.

5. Conclusion

An integrated and straightforward 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.

References

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