https://doi.org/10.1038/s41596-022-00785-5

## Supplementary information

# Comprehensive multi-attribute method workflow for biotherapeutic characterization and current good manufacturing practices testing

# Comprehensive multi-attribute method (MAM) workflow for biotherapeutic characterization and cGMP testing

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| | |
|---|---|
| Supplementary Figure 1 | Sequence coverage map of NISTmAb obtained using (a) in-solution tryptic digestion or (b) automated digestion |
| Supplementary Figure 2 | Sequence coverage map of a chimeric IgG1 DP obtained using (a) in-solution tryptic digestion or (b) automated digestion |
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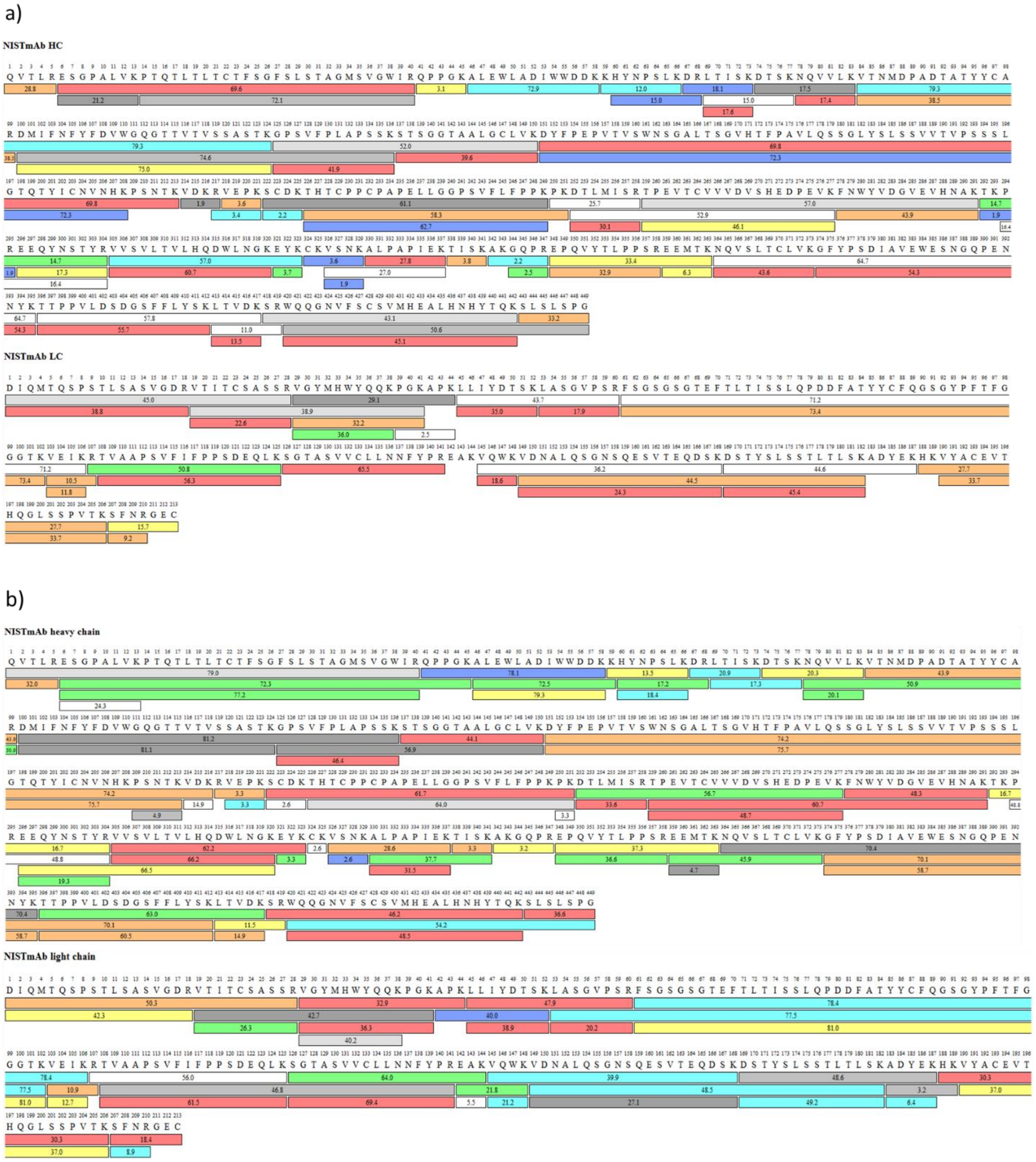
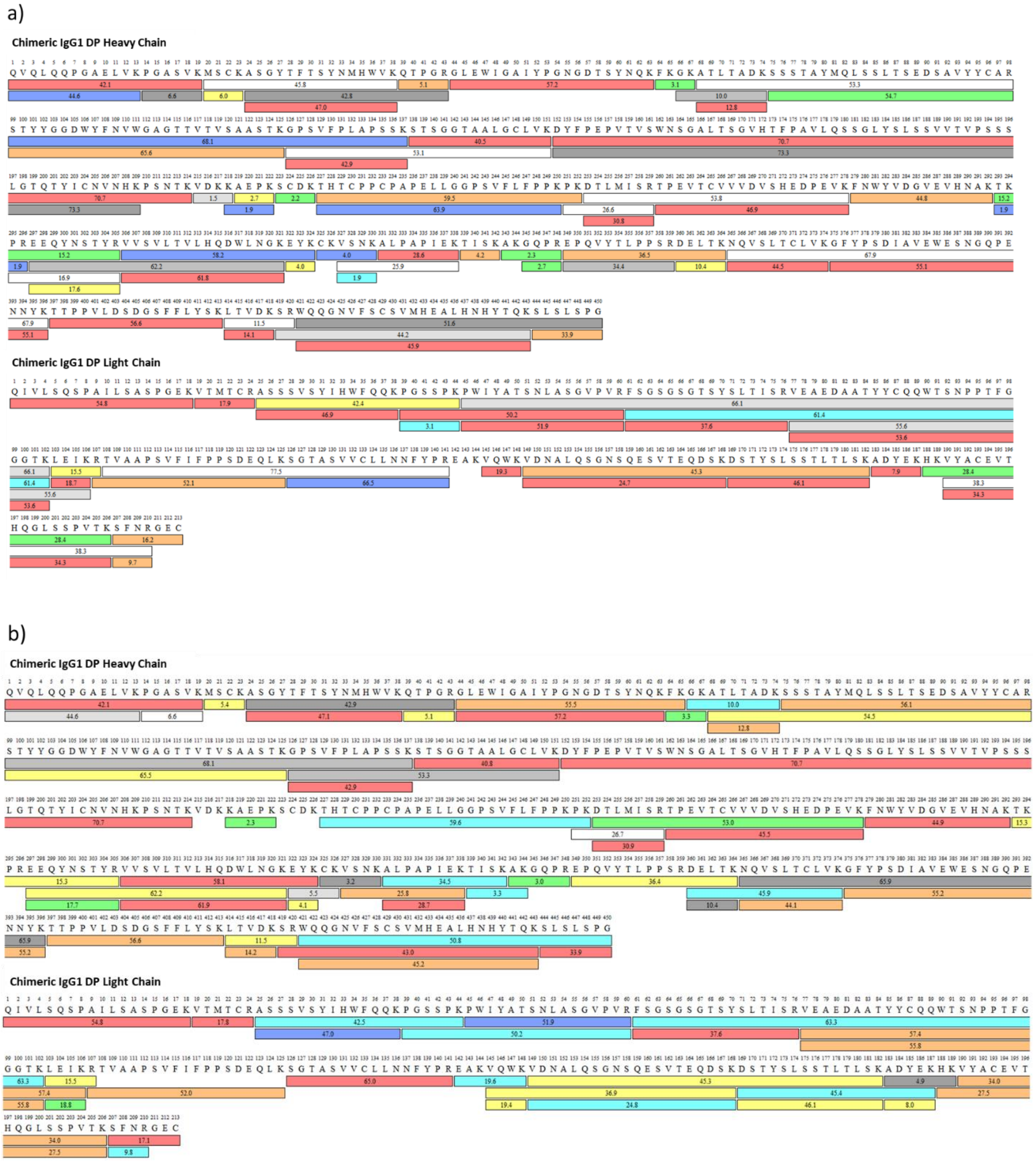
| Supplementary Table 1 | Summary of PTMs identified and quantified for NIST mAb for in-solution and automated trypsin digestion |

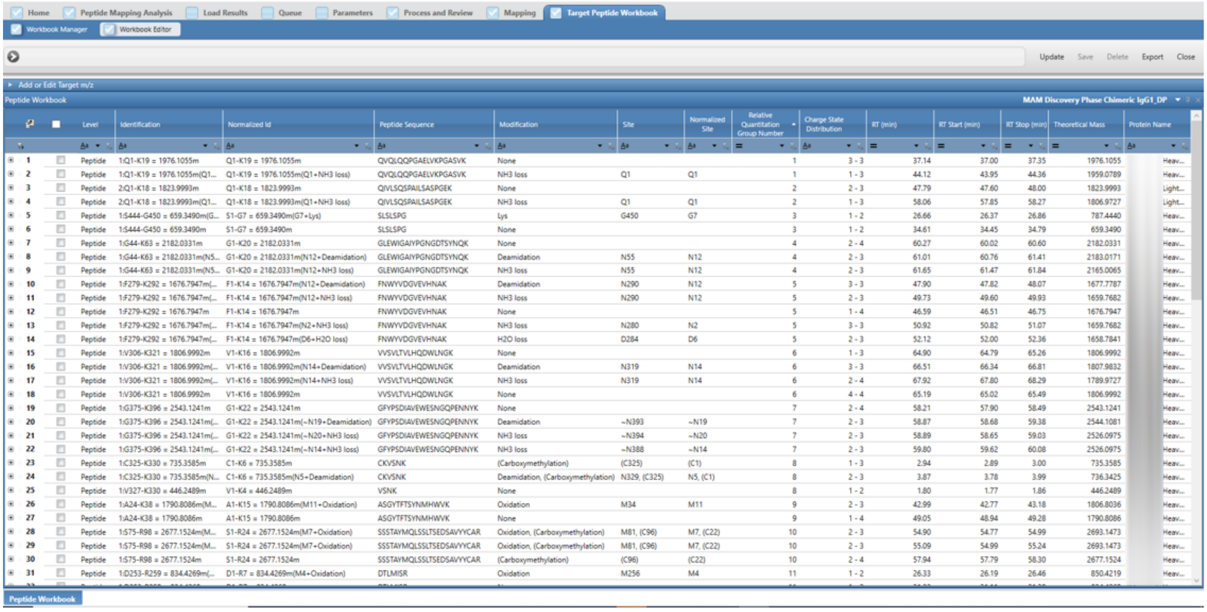
Supplementary Figure 1. Sequence coverage map of NISTmAb obtained using (a) in-solution tryptic digestion or (b) automated digestion. The coloured bars show the identified peptides, with the numbers in the bars reflecting the retention time. The different colors indicate the peptide recovery in the MS1 scan: red >50%, orange >20% and yellow >10% represent good recovery. Green, >5%, light blue >2% and cyan >1% represent fair recovery and grey-white scale shows poor recovered peptides.



Supplementary Figure 2. Sequence coverage map of chimeric IgG1 DP obtained using (a) in-solution tryptic digestion or (b) automated digestion. The coloured bars show the identified peptides, with the numbers in the bars reflecting the retention time. The different colors indicate the peptide recovery in the MS1 scan: red >50%, orange >20% and yellow >10% represent good recovery. Green, >5%, light blue >2% and cyan >1% represent fair recovery and grey-white scale shows poor recovered peptides.

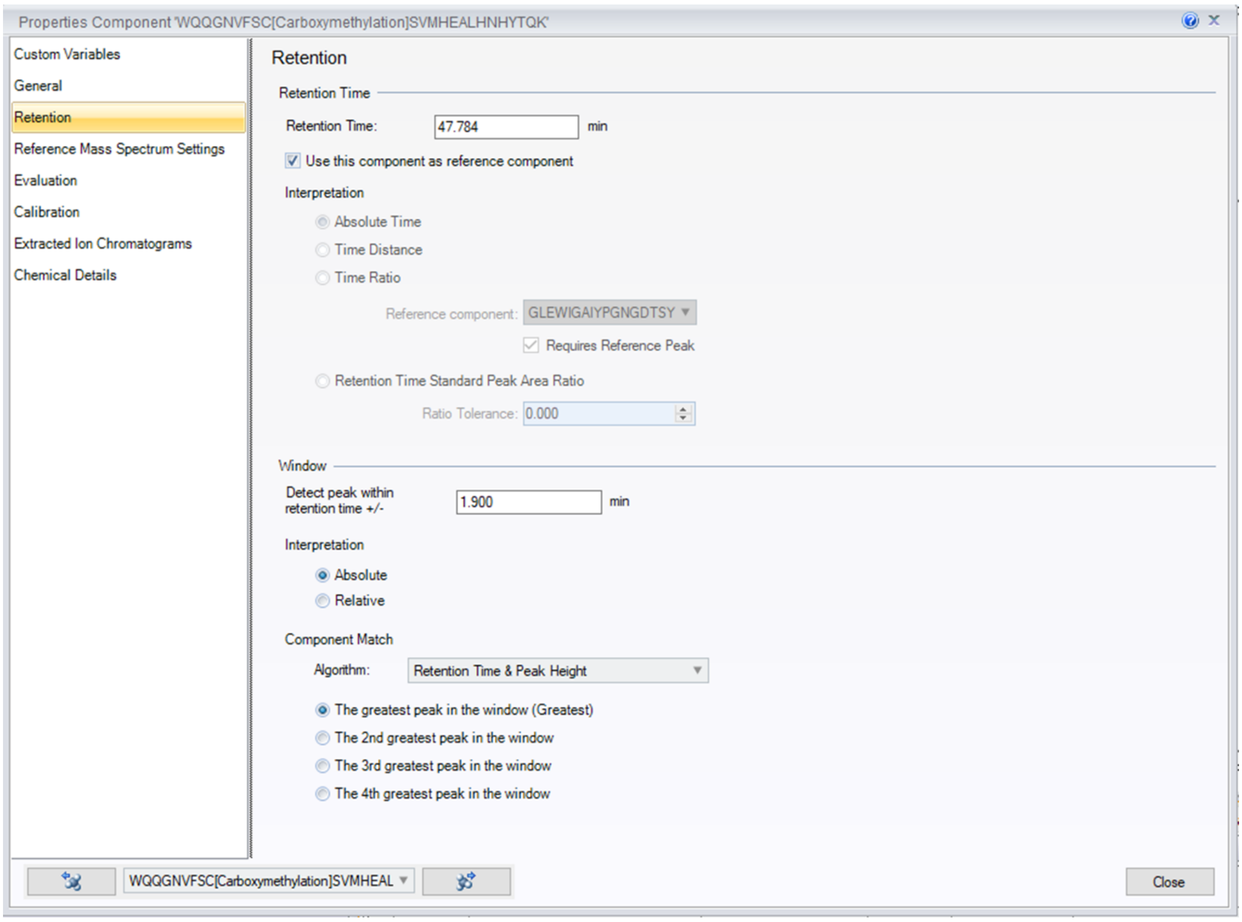


Supplementary Figure 3. BioPharma Finder software dashboard showing the created target peptide workbook during MAM discovery phase for chimeric IgG1 DP.



|  | ☐ | Level | Identification | Normalized Id | Peptide Sequence | Modification | Site | Normalized Site | Relative Quantitation Group Number | Charge State Distribution | RT (min) | RT Start (min) | RT Stop (min) | Theoretical Mass | Protein Name |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | ☐ | Peptide | 1:Q1-K19 = 1976.1055m | Q1-K19 = 1976.1055m | QVQLQQPGAELVKPGASVK | None |  |  | 1 | 3 - 3 | 37.14 | 37.00 | 37.35 | 1976.1055 | Heav... |
| 2 | ☐ | Peptide | 1:Q1-K19 = 1976.1055m(Q1... | Q1-K19 = 1976.1055m(Q1+NH3 loss) | QVQLQQPGAELVKPGASVK | NH3 loss | Q1 | Q1 | 1 | 1 - 3 | 44.12 | 43.95 | 44.36 | 1959.0789 | Heav... |
| 3 | ☐ | Peptide | 2:Q1-K18 = 1823.9993m | Q1-K18 = 1823.9993m | QIVLSQSPAILSASPGEK | None |  |  | 2 | 2 - 3 | 47.79 | 47.60 | 48.00 | 1823.9993 | Light... |
| 4 | ☐ | Peptide | 2:Q1-K18 = 1823.9993m(Q1... | Q1-K18 = 1823.9993m(Q1+NH3 loss) | QIVLSQSPAILSASPGEK | NH3 loss | Q1 | Q1 | 2 | 1 - 3 | 58.06 | 57.85 | 58.27 | 1806.9727 | Light... |
| 5 | ☐ | Peptide | 1:S444-G450 = 659.3490m(G... | S1-G7 = 659.3490m(G7+Lys) | SLSLSPG | Lys | G450 | G7 | 3 | 1 - 2 | 26.66 | 26.37 | 26.86 | 787.4440 | Heav... |
| 6 | ☐ | Peptide | 1:S444-G450 = 659.3490m | S1-G7 = 659.3490m | SLSLSPG | None |  |  | 3 | 1 - 2 | 34.61 | 34.45 | 34.79 | 659.3490 | Heav... |
| 7 | ☐ | Peptide | 1:G44-K63 = 2182.0331m | G1-K20 = 2182.0331m | GLEWIGAIYPGNGDTSYNQK | None |  |  | 4 | 2 - 4 | 60.27 | 60.02 | 60.60 | 2182.0331 | Heav... |
| 8 | ☐ | Peptide | 1:G44-K63 = 2182.0331m(N5... | G1-K20 = 2182.0331m(N12+Deamidation) | GLEWIGAIYPGNGDTSYNQK | Deamidation | N55 | N12 | 4 | 2 - 3 | 61.01 | 60.76 | 61.41 | 2183.0171 | Heav... |
| 9 | ☐ | Peptide | 1:G44-K63 = 2182.0331m(N5... | G1-K20 = 2182.0331m(N12+NH3 loss) | GLEWIGAIYPGNGDTSYNQK | NH3 loss | N55 | N12 | 4 | 2 - 3 | 61.65 | 61.47 | 61.84 | 2165.0065 | Heav... |
| 10 | ☐ | Peptide | 1:F279-K292 = 1676.7947m(... | F1-K14 = 1676.7947m(N12+Deamidation) | FNWYVDGVEVHNAK | Deamidation | N290 | N12 | 5 | 3 - 3 | 47.90 | 47.82 | 48.07 | 1677.7787 | Heav... |
| 11 | ☐ | Peptide | 1:F279-K292 = 1676.7947m(... | F1-K14 = 1676.7947m(N12+NH3 loss) | FNWYVDGVEVHNAK | NH3 loss | N290 | N12 | 5 | 2 - 3 | 49.73 | 49.60 | 49.93 | 1659.7682 | Heav... |
| 12 | ☐ | Peptide | 1:F279-K292 = 1676.7947m | F1-K14 = 1676.7947m | FNWYVDGVEVHNAK | None |  |  | 5 | 1 - 4 | 46.59 | 46.51 | 46.75 | 1676.7947 | Heav... |
| 13 | ☐ | Peptide | 1:F279-K292 = 1676.7947m(... | F1-K14 = 1676.7947m(N2+NH3 loss) | FNWYVDGVEVHNAK | NH3 loss | N280 | N2 | 5 | 3 - 3 | 50.92 | 50.82 | 51.07 | 1659.7682 | Heav... |
| 14 | ☐ | Peptide | 1:F279-K292 = 1676.7947m(... | F1-K14 = 1676.7947m(D6+H2O loss) | FNWYVDGVEVHNAK | H2O loss | D284 | D6 | 5 | 2 - 3 | 52.12 | 52.00 | 52.36 | 1658.7841 | Heav... |
| 15 | ☐ | Peptide | 1:V306-K321 = 1806.9992m | V1-K16 = 1806.9992m | VVSVLTVLHQDWLNGK | None |  |  | 6 | 1 - 3 | 64.90 | 64.79 | 65.26 | 1806.9992 | Heav... |
| 16 | ☐ | Peptide | 1:V306-K321 = 1806.9992m(... | V1-K16 = 1806.9992m(N14+Deamidation) | VVSVLTVLHQDWLNGK | Deamidation | N319 | N14 | 6 | 3 - 3 | 66.51 | 66.34 | 66.81 | 1807.9832 | Heav... |
| 17 | ☐ | Peptide | 1:V306-K321 = 1806.9992m(... | V1-K16 = 1806.9992m(N14+NH3 loss) | VVSVLTVLHQDWLNGK | NH3 loss | N319 | N14 | 6 | 2 - 4 | 67.92 | 67.80 | 68.29 | 1789.9727 | Heav... |
| 18 | ☐ | Peptide | 1:V306-K321 = 1806.9992m | V1-K16 = 1806.9992m | VVSVLTVLHQDWLNGK | None |  |  | 6 | 4 - 4 | 65.19 | 65.02 | 65.49 | 1806.9992 | Heav... |
| 19 | ☐ | Peptide | 1:G375-K396 = 2543.1241m | G1-K22 = 2543.1241m | GFYPSDIAVEWESNGQPENNYK | None |  |  | 7 | 2 - 4 | 58.21 | 57.90 | 58.49 | 2543.1241 | Heav... |
| 20 | ☐ | Peptide | 1:G375-K396 = 2543.1241m(... | G1-K22 = 2543.1241m(~N19+Deamidation) | GFYPSDIAVEWESNGQPENNYK | Deamidation | ~N393 | ~N19 | 7 | 2 - 3 | 58.87 | 58.68 | 59.38 | 2544.1081 | Heav... |
| 21 | ☐ | Peptide | 1:G375-K396 = 2543.1241m(... | G1-K22 = 2543.1241m(~N20+NH3 loss) | GFYPSDIAVEWESNGQPENNYK | NH3 loss | ~N394 | ~N20 | 7 | 2 - 3 | 58.89 | 58.65 | 59.03 | 2526.0975 | Heav... |
| 22 | ☐ | Peptide | 1:G375-K396 = 2543.1241m(... | G1-K22 = 2543.1241m(~N14+NH3 loss) | GFYPSDIAVEWESNGQPENNYK | NH3 loss | ~N388 | ~N14 | 7 | 2 - 3 | 59.80 | 59.62 | 60.08 | 2526.0975 | Heav... |
| 23 | ☐ | Peptide | 1:C325-K330 = 735.3585m | C1-K6 = 735.3585m | CKVSNK | (Carboxymethylation) | (C325) | (C1) | 8 | 1 - 3 | 2.94 | 2.89 | 3.00 | 735.3585 | Heav... |
| 24 | ☐ | Peptide | 1:C325-K330 = 735.3585m(N... | C1-K6 = 735.3585m(N5+Deamidation) | CKVSNK | Deamidation, (Carboxymethylation) | N329, (C325) | N5, (C1) | 8 | 2 - 3 | 3.87 | 3.78 | 3.99 | 736.3425 | Heav... |
| 25 | ☐ | Peptide | 1:V327-K330 = 446.2489m | V1-K4 = 446.2489m | VSNK | None |  |  | 8 | 1 - 2 | 1.80 | 1.77 | 1.86 | 446.2489 | Heav... |
| 26 | ☐ | Peptide | 1:A24-K38 = 1790.8086m(M... | A1-K15 = 1790.8086m(M11+Oxidation) | ASGYTFTSYNMHWVK | Oxidation | M34 | M11 | 9 | 2 - 3 | 42.99 | 42.77 | 43.18 | 1806.8036 | Heav... |
| 27 | ☐ | Peptide | 1:A24-K38 = 1790.8086m | A1-K15 = 1790.8086m | ASGYTFTSYNMHWVK | None |  |  | 9 | 1 - 4 | 49.05 | 48.94 | 49.28 | 1790.8086 | Heav... |
| 28 | ☐ | Peptide | 1:S75-R98 = 2677.1524m(M... | S1-R24 = 2677.1524m(M7+Oxidation) | SSSTAYMQLSSLTSEDSAVYYCAR | Oxidation, (Carboxymethylation) | M81, (C96) | M7, (C22) | 10 | 2 - 3 | 54.90 | 54.77 | 54.99 | 2693.1473 | Heav... |
| 29 | ☐ | Peptide | 1:S75-R98 = 2677.1524m(M... | S1-R24 = 2677.1524m(M7+Oxidation) | SSSTAYMQLSSLTSEDSAVYYCAR | Oxidation, (Carboxymethylation) | M81, (C96) | M7, (C22) | 10 | 2 - 3 | 55.09 | 54.99 | 55.24 | 2693.1473 | Heav... |
| 30 | ☐ | Peptide | 1:S75-R98 = 2677.1524m | S1-R24 = 2677.1524m | SSSTAYMQLSSLTSEDSAVYYCAR | (Carboxymethylation) | (C96) | (C22) | 10 | 2 - 4 | 57.94 | 57.79 | 58.30 | 2677.1524 | Heav... |
| 31 | ☐ | Peptide | 1:D253-R259 = 834.4269m(... | D1-R7 = 834.4269m(M4+Oxidation) | DTLMISR | Oxidation | M256 | M4 | 11 | 1 - 2 | 26.33 | 26.19 | 26.46 | 850.4219 | Heav... |

Supplementary Figure 4. Chromeleon CDS properties component window for unmodified peptide WQQGNVFSC[Carboxymethylation]SVMHEALHNHYTQK, which is selected as a reference component to monitor M431+oxidation.


Properties Component 'WQQGNVFSC[Carboxymethylation]SVMHEALHNHYTQK'

Retention

Retention Time

Retention Time: 47.784 min

☑ Use this component as reference component

Interpretation

● Absolute Time
○ Time Distance
○ Time Ratio

Reference component: GLEWIGAIYPGNGDTSY

☑ Requires Reference Peak

○ Retention Time Standard Peak Area Ratio

Ratio Tolerance: 0.000

Window

Detect peak within retention time +/- 1.900 min

Interpretation

● Absolute
○ Relative

Component Match

Algorithm: Retention Time & Peak Height

● The greatest peak in the window (Greatest)
○ The 2nd greatest peak in the window
○ The 3rd greatest peak in the window
○ The 4th greatest peak in the window

Supplementary Table 1. Summary of PTMs identified and quantified for NIST mAb for in-solution and automated trypsin digestion. Relative abundances are calculated based on triplicate analysis (n=3).

| Modification | Sequence | Relative Abundance (n=3) | |
|---|---|---|---|
| | | In-solution | Automated KF |
| HC N328+deamidation | VSNKALPAPIEK<br>CKVSNK<br>VSNK | 0.30 | 0.52 |
| HC~N387/N392+deamidation | GFYPSDIAVEWESNGQPENNYK | 0.79 | 0.90 |
| HC N387+succinimide | GFYPSDIAVEWESNGQPENNYK | 2.06 | 0.59 |
| HC M255+oxidation | DTLMISR | 1.26 | 1.98 |
| HC G450+Lys | SLSLPG | 12.99 | 13.88 |
| HC N300+A2G0F | EEQYNSTYR | 39.60 | 39.01 |
| HC N300+A2G1F | EEQYNSTYR | 39.36 | 39.38 |
| HC N300+A2G2F | EEQYNSTYR | 8.73 | 8.91 |