Analytical and Bioanalytical Chemistry

Electronic Supplementary Material

Interlaboratory study of an optimised peptide mapping workflow using automated trypsin digestion for monitoring monoclonal antibody product quality attributes

Silvia Millán-Martín, Craig Jakes, Sara Carillo, Tom Buchanan, Marc Guender, Dan Bach Kristensen, Trine Meiborg Sloth, Martin Ørgaard, Ken Cook, Jonathan Bones

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Table S1 Analytical Instrumentation used for the 4 Laboratories across Europe

	Site A (Ireland)	Site B (Denmark)	Site C (UK)	Site D (Switzerland)
UHPLC	Vanquish™ Flex Binary	Vanquish™ Duo Ternary	Vanquish™ Horizon Binary	Vanquish™ Flex Binary
HRMS	Q Exactive™ Plus	Q Exactive™ Plus	Q Exactive™ Plus	Q Exactive™ Plus
SMART Digest	Magnetic Beads KingFisher™ Duo	Magnetic Beads KingFisher™ Duo	Magnetic Beads KingFisher™ Duo	Manual Magnetic beads

Table S2 96-well plate scheme for trypsin digestion

Lane	Content	Volume (µL)
A	Low pH buffer	150
	Sample (100µg)	10
	TCEP (final concentration 5mM)	2
	Water	38
B	Tip Comb	
C	Empty	
D	Magnetic SMART™ Beads	15
	Bead Buffer (Low pH buffer)	100
E	Bead Wash Buffer (Bead Buffer 1:4 (v/v))	200
F	Waste Lane (Water)	250

Table S3 BPF software parameter settings for peptide mapping data analysis

Component Detection	**Setting**
Absolute MS Signal Threshold	1.0 x 10^4 counts
Typical chromatographic peak width	0.3
Relative MS signal threshold (% base peak)	1
Relative Analog threshold (% of highest peak)	1
Width of Gaussian filter (represented as 1/n of chromatographic peak width)	3
Minimum valley to be considered as two chromatographic peaks	80.0%
Minimum MS peak width (Da)	1.2
Maximum MS peak width (Da)	4.2
Mass tolerance (ppm for high-res or Da for low-res)	4.00
Maximum retention time shift (min)	1.00
Maximum mass (Da)	30,000
Mass Centroiding Cutoff (% from base)	15
Identification	**Setting**
Maximum peptide mass	7,000
Mass Accuracy	5ppm
Minimum Confidence	0.8
Maximum Number of Modifications for a Peptide	1
Unspecified Modification	-58 to +162 Da
N-Glycosylation	CHO
Protease Specificity	High
Static Modifications	**Setting**
Side Chain	-
Variable Modifications	**Setting**
N Terminal	Gln - > Pyro Glu
C Terminal	Loss of lysine
Side Chain	Deamidation (N) Deamidation(Q) Glycation (K) Oxidation (MW) AsnAsu (N) AspAsu (D)

Table S4 Chromeleon™ Data System 7.2.9 parameter settings for peptide mapping data processing and PTMs quantitation

MS Detection	**Setting**
Extracted Ion Chromatogram	MS Default Detection Settings
Detection Algorithm	ICIS
Area noise factor	5
Peak noise factor	10
Baseline window	40
Noise method	INCOS
Min peak width	3
Multiplet resolution	10
Area tail extension	5
Area scan window	0
MS Component table	**Setting**
Supplementary Table S5 (.xlsx file)	.wbpf workbook from BPF3.1
MS Settings	**MS Chromatogram Settings**
Mass Precision	5 decimal places
Mass Tolerance (manually defined)	8.0 ppm
Smoothing	none
Peptide Table	**Setting**
Imported from BPF3.1 peptide workbook	-
Composite Scoring	**Setting**
Pass score if at least	2 criteria passed
Fail score if less than	1 criteria passed
MS Criteria	General MS
Isotopic dot product	≥ 0.9000
Mass accuracy	≤ 5.00 ppm
Peal apex alignment	≤ 0.50 min

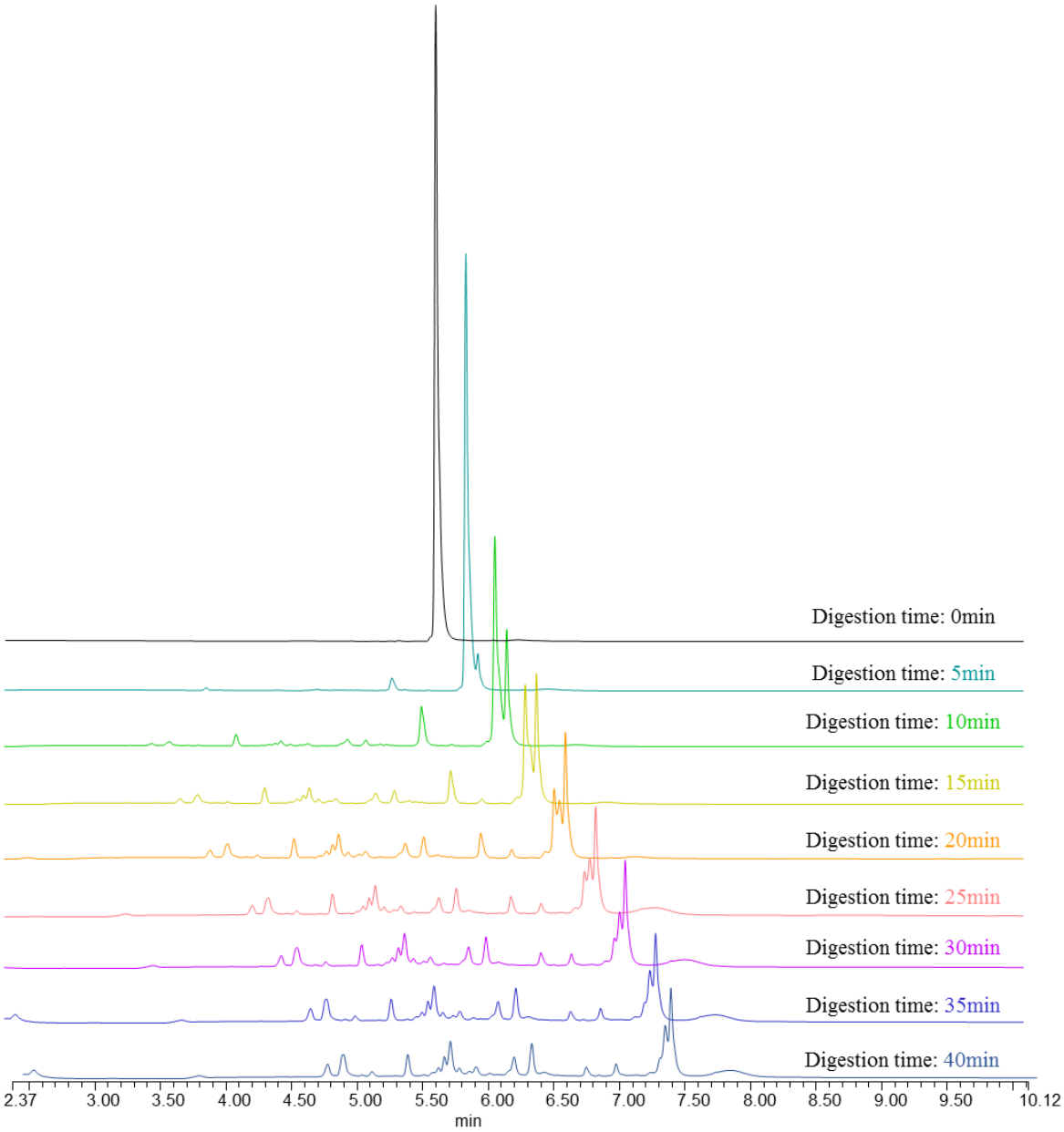

Fig. S1 UV Chromatogram traces (280nm) for trastuzumab RP-UV-MS Intact analysis during digestion time course study using buffer 1

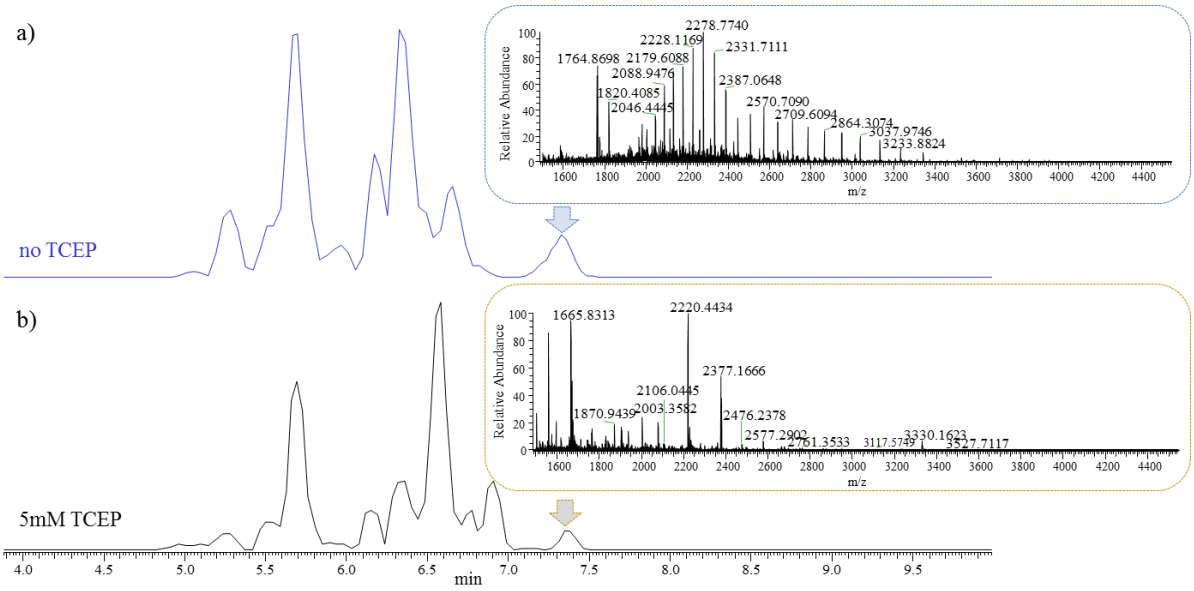

Fig. S2 Base Peak Chromatograms (BPC) for trastuzumab RP-UV-MS Intact Analysis using 5mM TCEP as reducing agent (a), and without TCEP addition (b). Digestion was performed for 35min and buffer 2

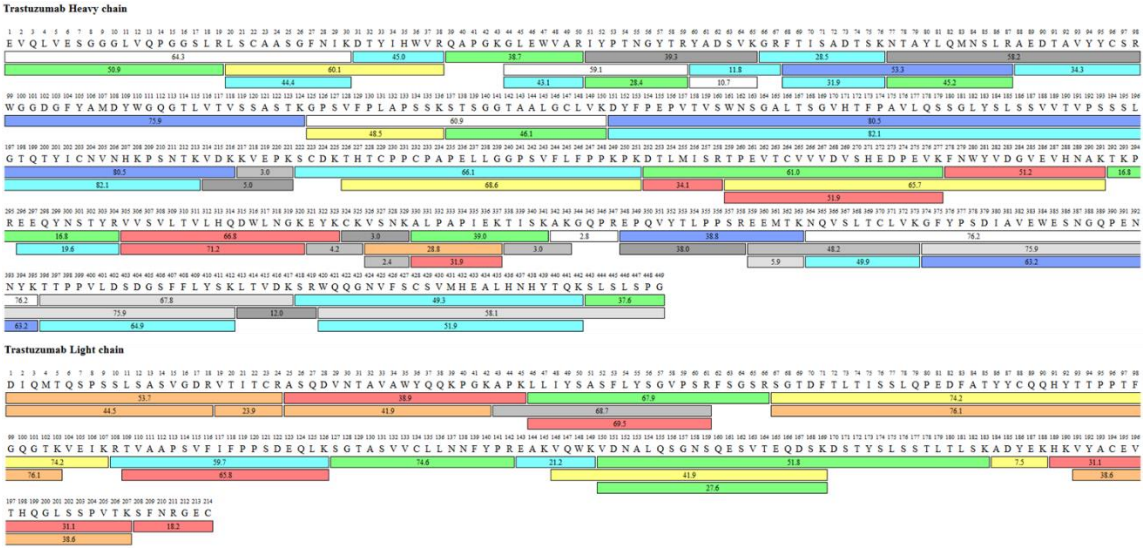

Fig. S3 Sequence coverage map of trastuzumab heavy (upper panel) and light (lower panel) chains, obtained using automated tryptic digestion with buffer 1 after 5 minutes. The coloured bars show the identified peptides, with the numbers in the bars reflecting the retention time. The different colors indicate 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

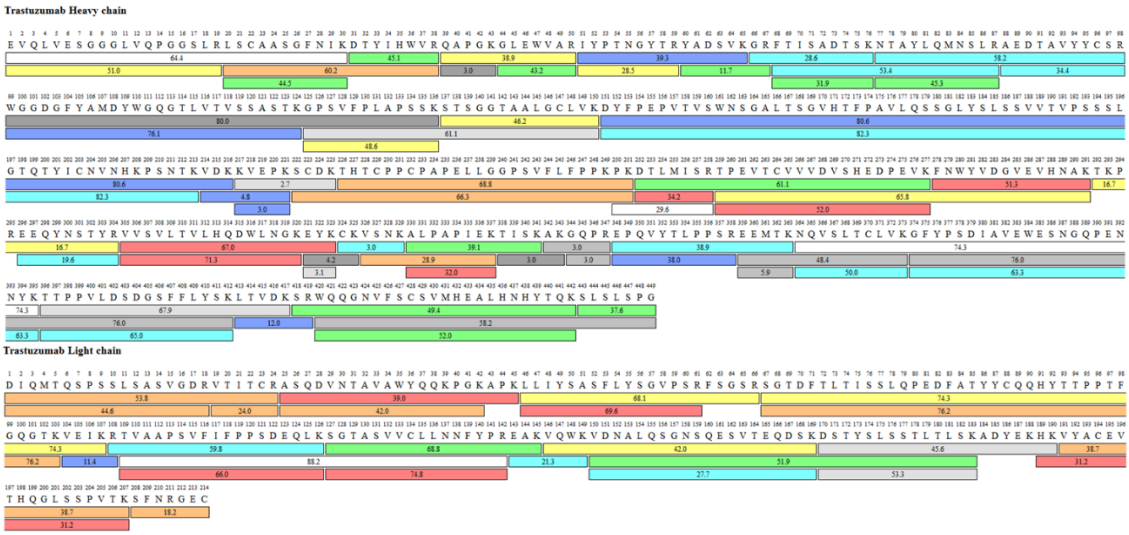

Fig. S4 Sequence coverage map of trastuzumab heavy (upper panel) and light (lower panel) chains, obtained using automated tryptic digestion with buffer 2 after 5 minutes. The coloured bars show the identified peptides, with the numbers in the bars reflecting the retention time. The different colors indicate 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

Table S6 Sequence coverage for the time course of digestion for trastuzumab

Proteins		Avg Number of MS Peaks (n=3)	Average Sequence Coverage (n=3)
Buffer 1, 5min	HC	353	100%
	LC	103	100%
Buffer 1, 10min	HC	449	100%
	LC	134	100%
Buffer 1, 15min	HC	510	100%
	LC	152	100%
Buffer 1, 20min	HC	578	100%
	LC	167	100%
Buffer 1, 25min	HC	570	100%
	LC	161	100%
Buffer 1, 30min	HC	553	100%
	LC	158	100%
Buffer 1, 35min	HC	536	100%
	LC	147	100%
Buffer 1, 40min	HC	594	100%
	LC	180	100%
Buffer 2, 5min	HC	353	100%
	LC	95	100%
Buffer 2, 10min	HC	548	100%
	LC	156	100%
Buffer 2, 15min	HC	593	100%
	LC	169	100%
Buffer 2, 20min	HC	617	100%
	LC	172	100%
Buffer 2, 25min	HC	621	100%
	LC	165	100%
Buffer 2, 30min	HC	599	100%
	LC	155	100%
Buffer 2, 35min	HC	555	100%
	LC	149	100%
Buffer 2, 40min	HC	649	100%
	LC	172	100%

Table S7 Summary of PTMs identified and quantified for trastuzumab after digestion with trypsin beads, buffer 1 and 5mM TCEP addition on the KingFisher™ Duo Prime system

Modification	5min (n=3)	10min (n=3)	15min (n=3)	20min (n=3)	25min (n=3)	30min (n=3)	35min (n=3)	40min (n=3)
HC N55+Deamidation	—	0.63	0.61	0.98	1.17	1.78	2.23	3.64
HC N77+Deamidation	0.07	0.04	0.06	0.12	0.16	0.24	0.25	0.26
HC N84+Deamidation	0.02	0.02	0.04	0.07	0.07	0.12	0.12	0.12
HC N289+Deamidation	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
HC N318+Deamidation	0.05	0.11	0.05	0.22	0.16	0.28	0.90	0.26
HC N364+Deamidation	0.01	0.04	0.02	0.12	0.09	0.09	0.33	0.44
LC N30+Deamidation	6.82	8.41	8.24	8.89	8.94	9.23	9.80	9.12
LC ~N137+Deamidation	0.88	0.30	0.21	0.72	1.26	3.73	1.30	1.53
LC N210+Deamidation	0.00	0.00	0.00	0.00	0.01	0.02	0.02	0.07
HC M255+Oxidation	1.36	1.06	1.11	1.02	1.13	1.24	1.10	1.25
HC M431+Oxidation	0.07	0.19	0.19	0.18	0.21	0.14	0.22	0.15
HC G449+Lys	1.08	1.44	1.60	1.62	1.66	1.60	1.83	1.69
HC N55+AsnAsu	1.00	1.21	1.45	2.14	2.36	2.93	3.39	3.60
HC N318+AsnAsu	0.48	1.05	1.74	2.77	3.38	3.93	3.41	3.90
LC N30+AsnAsu	0.19	1.48	1.53	1.08	0.87	0.50	0.50	0.31
LC ~N138+AsnAsu	9.67	1.49	1.71	3.06	3.87	7.43	2.04	1.92

Table S7 (continued) Summary of PTMs identified and quantified for trastuzumab after digestion with trypsin beads, buffer 1 and 5mM TCEP addition on the KingFisher™ Duo Prime system

Modification	5min (n=3)	10min (n=3)	15min (n=3)	20min (n=3)	25min (n=3)	30min (n=3)	35min (n=3)	40min (n=3)
HC ~D102+AspAsu	0.21	0.48	0.73	0.92	1.10	1.52	1.27	1.47
HC ~D283+AspAsu	0.53	1.49	2.57	2.68	2.57	4.02	3.64	4.54
HC ~D404+AspAsu	0.28	0.03	0.01	1.43	1.64	1.56	1.29	1.033
HC D224+isomerization	69.11	55.64	70.37	52.95	66.25	66.19	75.22	84.46
HC N300+A1G0	2.07	2.35	2.16	2.15	2.10	2.10	2.00	2.16
HC N300+A1G0F	11.43	8.76	7.43	7.28	6.40	5.48	7.27	6.32
HC N300+A1G1F	3.26	1.99	1.75	1.76	1.45	1.19	1.64	1.78
HC N300+A2G0	3.78	5.16	5.46	5.56	5.69	5.86	5.62	5.82
HC N300+A2G0F	48.99	50.91	52.15	52.18	53.23	54.56	52.07	51.93
HC N300+A2G1F	22.04	22.69	23.41	23.44	23.50	23.19	23.86	23.27
HC N300+A2G2F	2.00	1.84	1.91	1.75	1.80	1.79	2.16	2.17
HC N300+M5	2.17	2.11	2.08	2.15	2.16	2.29	2.05	2.26
HC N300+Unglycosylated	4.26	4.18	3.63	3.73	3.65	3.53	3.32	4.28

Table S8 Summary of PTMs identified and quantified for trastuzumab after digestion with trypsin beads, buffer 2 and 5mM TCEP addition on the KingFisher™ Duo Prime system

Modification	5min (n=3)	10min (n=3)	15min (n=3)	20min (n=3)	25min (n=3)	30min (n=3)	35min (n=3)	40min (n=3)
HC N55+Deamidation	0.64	0.48	0.85	1.04	1.69	1.95	3.14	3.58
HC N77+Deamidation	0.01	0.02	0.08	0.13	0.15	0.19	0.29	0.36
HC N84+Deamidation	0.14	0.02	0.05	0.07	0.09	0.09	0.08	0.15
HC N289+Deamidation	0.01	0.02	0.04	0.05	0.14	0.075	0.04	0.09
HC N318+Deamidation	0.05	0.43	0.28	1.17	0.62	1.43	1.023	3.26
HC N364+Deamidation	0.00	0.01	0.01	0.13	0.21	0.13	0.42	0.47
LC N30+Deamidation	6.92	8.90	8.88	9.65	9.34	9.58	10.06	9.40
LC ~N137+Deamidation	0.00	0.27	0.62	1.11	2.10	5.00	1.33	2.60
LC N210+Deamidation	0.00	0.00	0.00	0.01	0.02	0.03	0.04	0.07
HC M255+Oxidation	1.18	0.48	0.47	0.43	0.48	0.85	0.29	0.26
HC M431+Oxidation	0.09	0.17	0.17	0.15	0.15	0.14	0.17	0.17
HC G449+Lys	0.57	1.44	1.53	1.63	1.63	1.56	1.81	1.76
HC N55+AsnAsu	1.30	1.22	1.89	2.29	2.73	3.24	3.57	3.52
HC N318+AsnAsu	0.51	1.52	3.03	6.31	5.49	9.19	3.90	4.44
LC N30+AsnAsu	0.31	1.32	0.89	0.73	0.48	0.39	0.27	0.21
LC ~N137+AsnAsu	0.00	0.00	0.02	0.02	0.00	0.00	0.00	0.00

Table S8 (continued) Summary of PTMs identified and quantified for trastuzumab after digestion with trypsin beads, buffer 2 and 5mM TCEP addition on the KingFisher™ Duo Prime system.

Modification	5min (n=3)	10min (n=3)	15min (n=3)	20min (n=3)	25min (n=3)	30min (n=3)	35min (n=3)	40min (n=3)
HC ~D102+AspAsu	0.37	0.16	0.80	0.81	0.96	1.38	1.14	1.11
HC ~D283+AspAsu	0.90	0.46	4.02	6.45	5.28	6.92	3.57	4.54
HC ~D404+AspAsu	0.03	1.32	2.02	2.21	1.13	1.84	0.68	1.90
HC D224+isomerization	0.03	52.43	45.51	45.68	64.12	54.92	49.51	47.36
HC N300+A1G0	1.98	2.35	2.15	2.07	2.10	2.10	2.11	2.04
HC N300+A1G0F	7.54	10.03	7.75	7.12	6.38	5.43	7.56	6.73
HC N300+A1G1F	1.85	2.35	1.51	1.72	1.34	1.16	1.52	1.17
HC N300+A2G0	4.48	5.03	5.29	5.42	5.64	5.93	5.29	5.51
HC N300+A2G0F	53.65	50.11	52.68	52.89	53.19	54.71	52.33	52.80
HC N300+A2G1F	23.28	22.74	23.59	23.64	23.99	23.17	23.82	24.43
HC N300+A2G2F	1.77	2.05	2.15	2.18	2.17	1.98	2.22	2.27
HC N300+M5	1.99	2.01	2.03	2.06	2.16	2.26	2.02	2.14
HC N300+Unglycosylated	3.45	3.33	2.84	2.89	3.02	3.26	3.12	2.90

Table S10 Analysis of Variance (ANOVA) to measure intra and inter-lab precision for NISTmAb complete compliant peptide mapping workflow

HC N328+Deam

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.1001	3	0.0334	153.9872	2.05E-07	4.0662
Within Groups	0.0017	8	0.0002			
Intra-Lab precision (RSD)	**10.22**					
Inter-Lab precision (RSD)	**44.43**					

HC N364+Deam

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.1659	3	0.0553	49.1556	1.69E-05	4.0662
Within Groups	0.0090	8	0.0011			
Intra-Lab precision (RSD)	**42.16**					
Inter-Lab precision (RSD)	**101.64**					

HC ~N392/N387+ Deam

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.7405	3	0.2468	219.4049	5.09E-08	4.0662
Within Groups	0.0090	8	0.0011			
Intra-Lab precision (RSD)	**20.22**					
Inter-Lab precision (RSD)	**105.25**					

HC D283+Succ

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	8.3645	3	2.7882	420.8549	3.86E-09	4.0662
Within Groups	0.0530	8	0.0066			
Intra-Lab precision (RSD)	**8.61**					
Inter-Lab precision (RSD)	**62.24**					

HC N318+Succ

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.7734	3	0.2578	52.6100	1.31E-05	4.0662
Within Groups	0.0392	8	0.0049			
Intra-Lab precision (RSD)	**8.28**					
Inter-Lab precision (RSD)	**20.68**					

HC ~N392/N387+Succ

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.8415	3	0.2805	125.5970	4.56E-07	4.0662
Within Groups	0.0179	8	0.0022			

Intra-Lab precision (RSD)	**5.54**
Inter-Lab precision (RSD)	**21.73**

Table S10 (continued) Analysis of Variance (ANOVA) to measure intra and inter-lab precision for NISTmAb complete compliant peptide mapping workflow

HC M255+Oxid

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.2813	3	0.0938	468.8194	2.51E-09	4.0662
Within Groups	0.0016	8	0.0002			
Intra-Lab precision (RSD)	**3.54**					
Inter-Lab precision (RSD)	**27.04**					

HC K450 Lys Loss

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	21.0218	3	7.0073	225.5554	4.57E-08	4.0662
Within Groups	0.2485	8	0.0311			
Intra-Lab precision (RSD)	**0.56**					
Inter-Lab precision (RSD)	**2.96**					

HC Q1+Gln->Pyro-Glu

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.4365	3	0.1455	831.3651	2.57E-10	4.0662
Within Groups	0.0014	8	0.0002			
Intra-Lab precision (RSD)	**0.04**					
Inter-Lab precision (RSD)	**0.38**					

HC N300+M5

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.6293	3	0.2098	143.0152	2.74E-07	4.0662
Within Groups	0.0117	8	0.0015			
Intra-Lab precision (RSD)	**8.14**					
Inter-Lab precision (RSD)	**34.11**					

Table S10 (continued) Analysis of Variance (ANOVA) to measure intra and inter-lab precision for NISTmAb complete compliant peptide mapping workflow

HC N300+A1G0F

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	94.4941	3	31.4980	438.9969	3.26E-09	4.0662
Within Groups	0.5740	8	0.0718			
Intra-Lab precision (RSD)	**9.10**					
Inter-Lab precision (RSD)	**67.20**					

HC N300+A2G0F

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	36.8955	3	12.2985	55.2535	1.09E-05	4.0662
Within Groups	1.7807	8	0.2226			
Intra-Lab precision (RSD)	**3.43**					
Inter-Lab precision (RSD)	**8.79**					

HC N300+A1G1F

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	14.1954	3	4.7318	183.6998	1.03E-07	4.0662
Within Groups	0.2061	8	0.0258			
Intra-Lab precision (RSD)	**9.18**					
Inter-Lab precision (RSD)	**43.65**					

HC N300+A2G1F

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	38.3946	3	12.7982	196.0907	7.93E-08	4.0662
Within Groups	0.5221	8	0.0653			
Intra-Lab precision (RSD)	**2.01**					
Inter-Lab precision (RSD)	**9.90**					

HC N300+A2G2F

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4.7004	3	1.5668	104.6852	9.28E-07	4.0662
Within Groups	0.1197	8	0.0150			
Intra-Lab precision (RSD)	**4.65**					
Inter-Lab precision (RSD)	**16.62**					

HC N300+ A2Ga1G1F

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.3505	3	0.1168	175.2333	1.23E-07	4.0662
Within Groups	0.0053	8	0.0007			
Intra-Lab precision (RSD)	**6.24**					
Inter-Lab precision (RSD)	**28.99**					