

Supporting Information

Enhanced Characterization of Lysine-Linked Antibody Drug Conjugates Enabled by Middle-Down Mass Spectrometry and HCD-Triggered EThcD and UVPD

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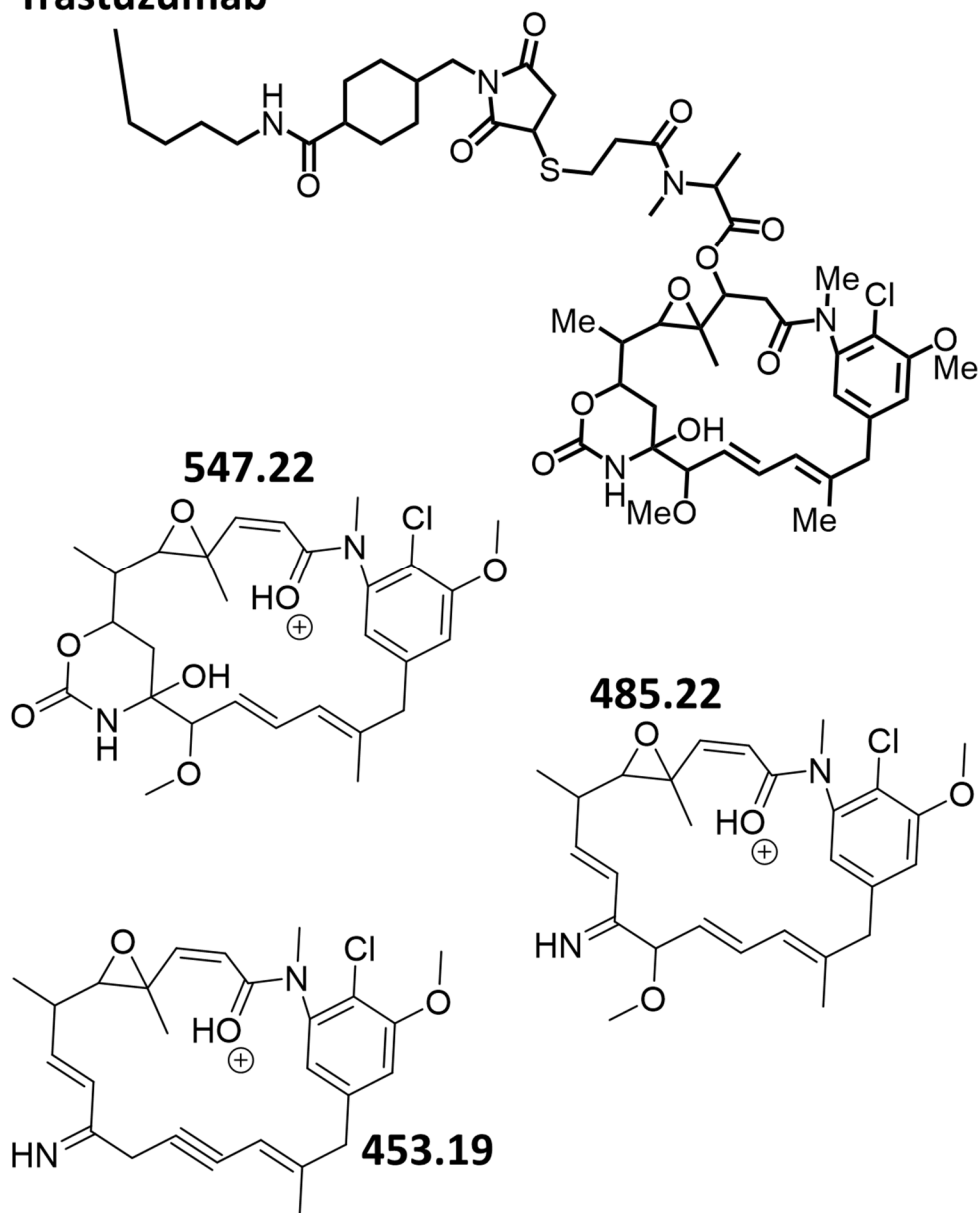
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Trastuzumab



Scheme S1. Structure of DM1 payload conjugated to trastuzumab and possible fragment ion structures that correspond to payload reporter ions of m/z 547.22, 485.22, and 453.19 Da observed in the HCD mass spectra.

30k Resolution: 34% sequence coverage (HC-K395 and HC-K417)

N G F Y P S D I A V E W E S N G Q P E N N Y K T T P 25
26 P V L D S D G S F F L Y S K L T V D K S R W Q Q G 50
51 N V F S C S V M H E A L L H N H Y T Q K C

240k Resolution: 37% sequence coverage (HC-K395 and HC-K417)

N G F Y P S D I A V E W E S N G Q P E N N Y K T T P 25
26 P V L D S D G S F F L Y S K L T V D K S R W Q Q G 50
51 N V F S C S V M H E A L L H N H Y T Q K C

Figure S1. Sequence coverage maps of an 9.8 kDa peptide (6+) for HCD with (A) 30,000 and (B) 240,000 resolution at m/z 200. K22 and K44 of the peptide sequence shown here corresponds to K395 and K417 of the antibody heavy chain. The modified lysine sites are shaded in gold.

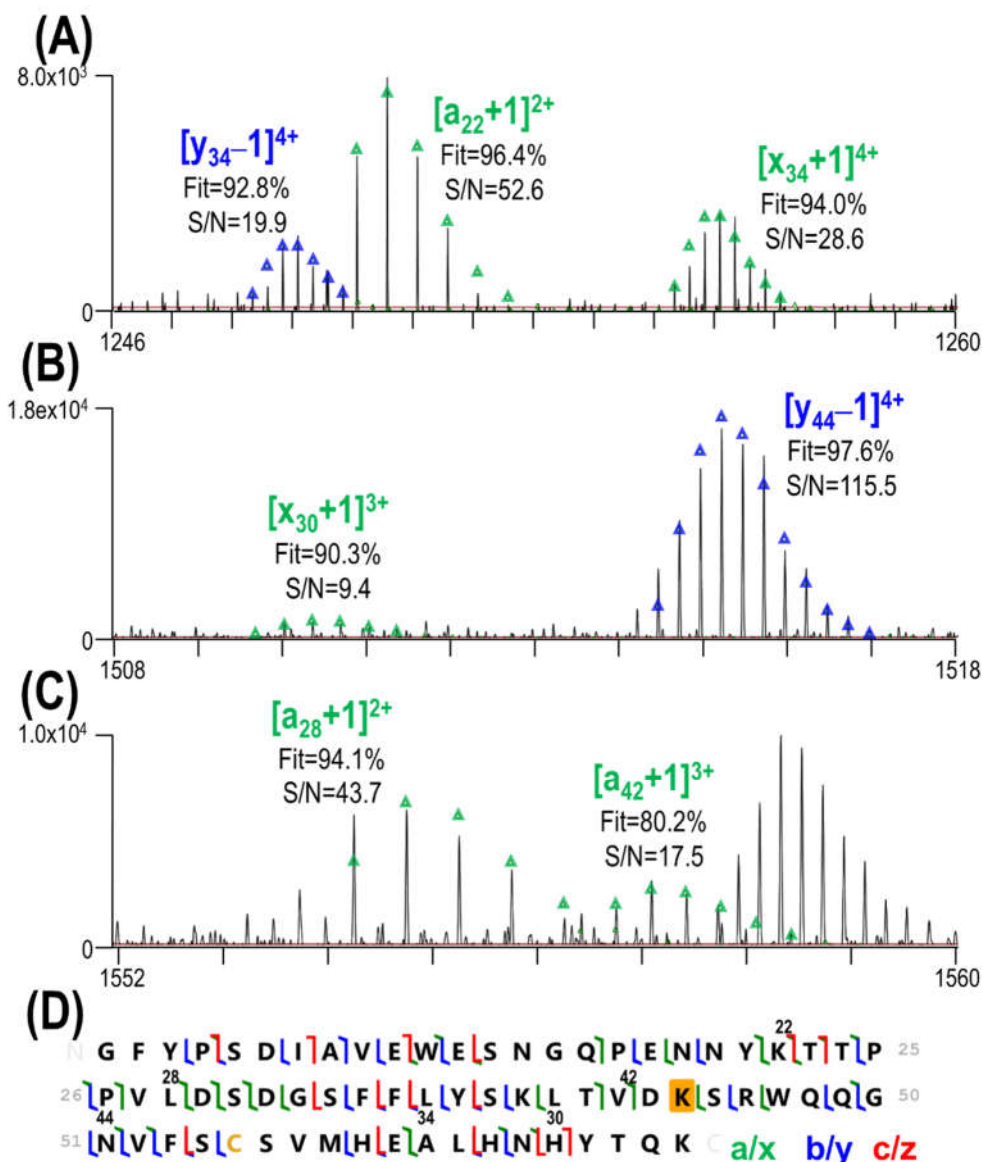


Figure S2. Expanded regions of the UVPD mass spectrum shown in **Figure 3C** illustrating examples of fragment ion isotope patterns with (A) high fit factors and signal to noise, (B) low signal to noise, and (C) low fit factors. (D) Sequence map highlighting the backbone cleavage sites from which the identified fragment ions originated. In this and other sequence maps, backbone cleavages that lead to a/x (green), b/y (blue) and c/z (green) ions are overlaid, partially obscuring the color coded cleavage sites. The modified lysine sites are shaded in gold.

A variety of fragment ions were identified with and without the payload, including C-terminal payload containing fragments (A) $[y_{34}-1]^{4+}$, (A) $[x_{34}+1]^{4+}$, (B) $[x_{30}+1]^{3+}$, and (B) $[y_{44}-1]^{4+}$, and N-terminal fragments without the payload (A) $[a_{22}+1]^{2+}$, (C) $[a_{28}+1]^{2+}$, and (C) $[a_{42}+1]^{3+}$. Fit factors are calculated in Thermo Xtract and signal to noise values and the theoretical isotopic distributions are generated with TDValidator.

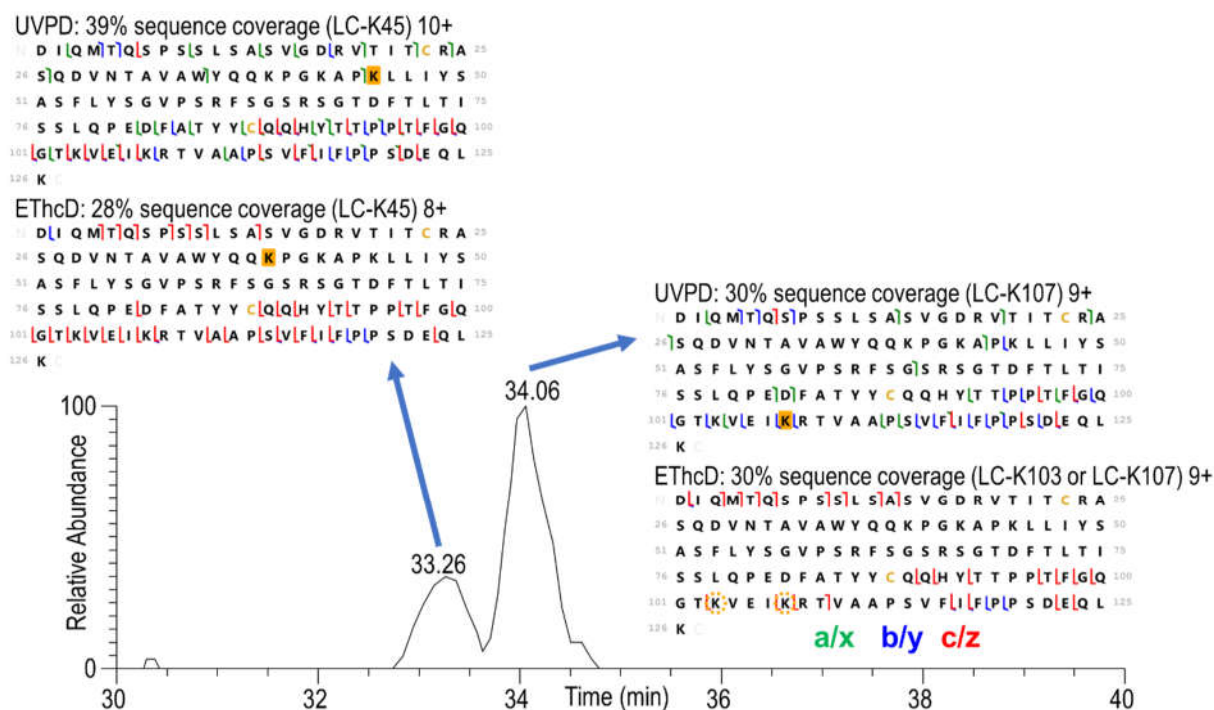


Figure S3. Extracted ion chromatogram revealing two light chain peptides (14.7 kDa, 8+), each containing 126 residues (D1 through K126) and a single payload. Sequence coverage maps obtained by EThcD and UVPD localize the payload to K45 (peptide at 33.26 min) or K107 (peptide at 34.06 min). The payload localization sites are shaded in gold when unambiguous and circled when ambiguous.

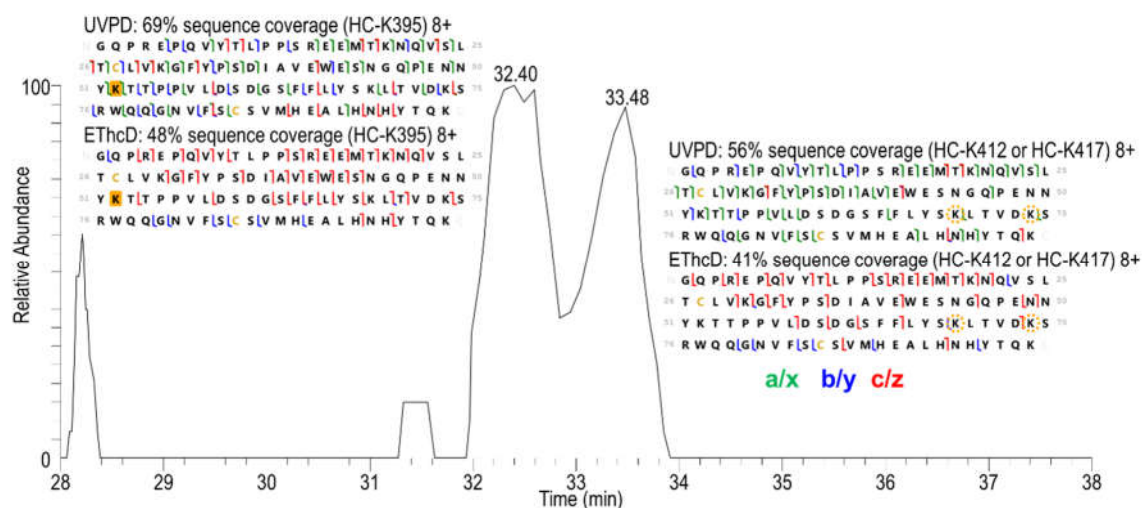


Figure S4. Extracted ion chromatogram revealing two heavy chain peptides (12.3 kDa, 8+), each containing 99 residues (G344 through K442) and a single payload. Sequence coverage maps

obtained by EThcD and UVPD localize the payload to HC-K395 (peptide at 32.40 min) or HC-K412/K417 (peptide at 33.48 min). K52, K69, and K74 of the sequence correspond to K395, K412, and K417 of the heavy chain. The payload localization sites are shaded in gold when unambiguous and circled when ambiguous.

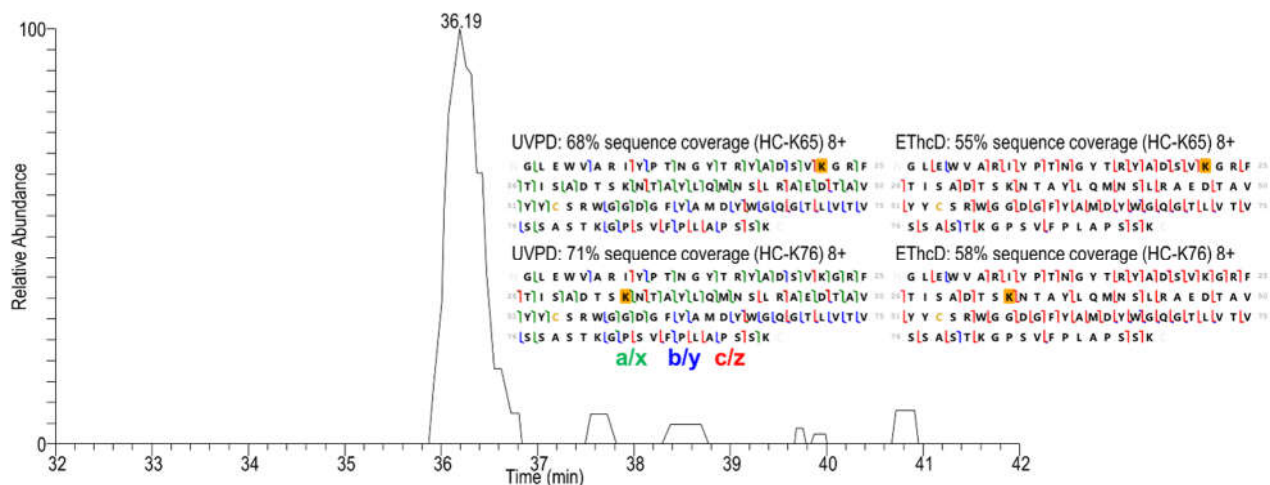


Figure S5. Extracted ion chromatogram of a heavy chain peptide (11.2 kDa, 8+) containing G44 through K136 and a single payload. Sequence coverage maps are included for EThcD and UVPD which localize the payload to both K65 and K76, although they are not chromatically resolved and are therefore co-isolated. K22 and K33 of the sequence correspond to K65 and K76 of the heavy chain. The payload sites are shaded in gold when unambiguously localized.

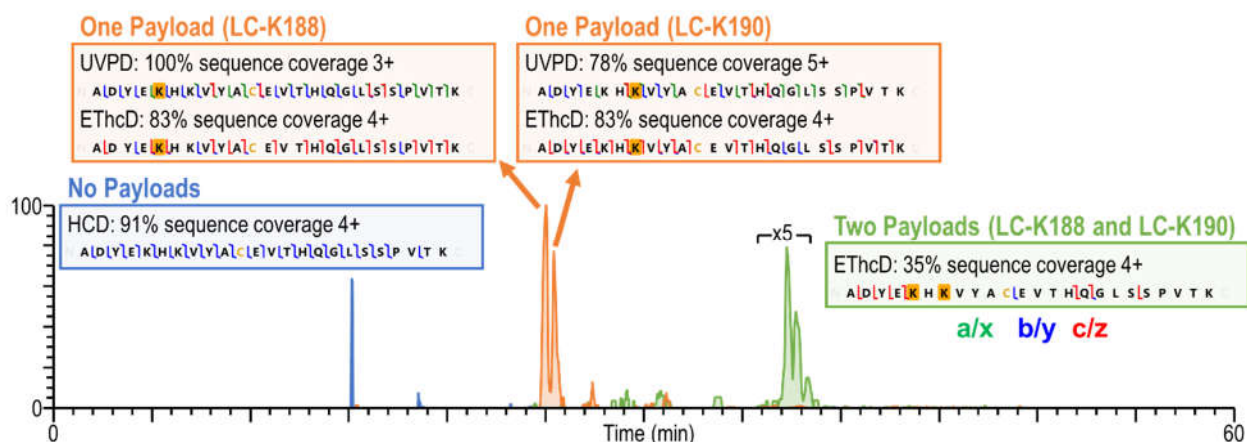


Figure S6. EICs for the 4+ charge state of a 24 amino acid long light chain peptide containing A184 through K207 with zero (m/z 673.33), one (m/z 913.18) or two (m/z 1152.27) payload conjugations. Sequence coverage maps are included for each chromatographic peak observed in

ambiguous. K24, K26, and K52 on these maps correspond to K249, K251, and K277 on the heavy chain. Owing to co-elution, two maps are included for peptide corresponding to the light chain containing one payload (t: 35 min).

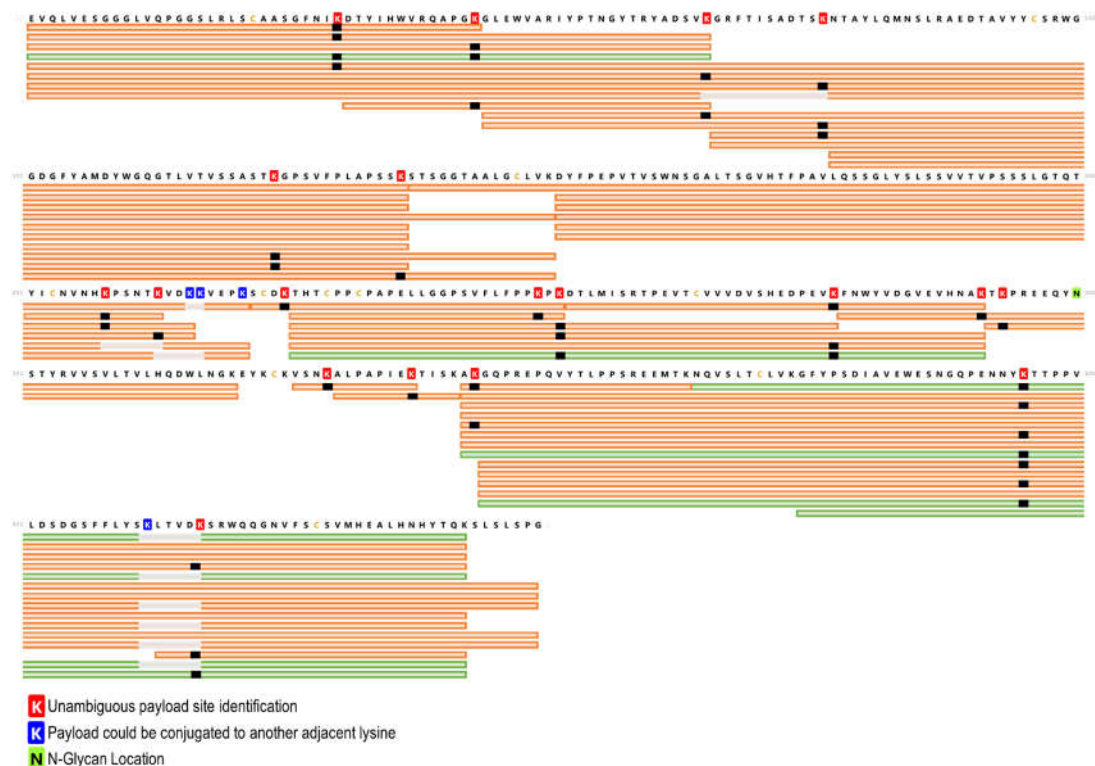


Figure S9. Peptide map displaying the global sequence coverage for the heavy chain. The same legend used in Figure 5 was retained for the heavy chain sequence. Peptides displayed in orange contain a single payload conjugation and peptides displayed in green contain two payload conjugations. Unambiguously localized payload conjugations sites are shown in black on for each peptide, and payloads that could be localized two multiple sites are shown in gray.

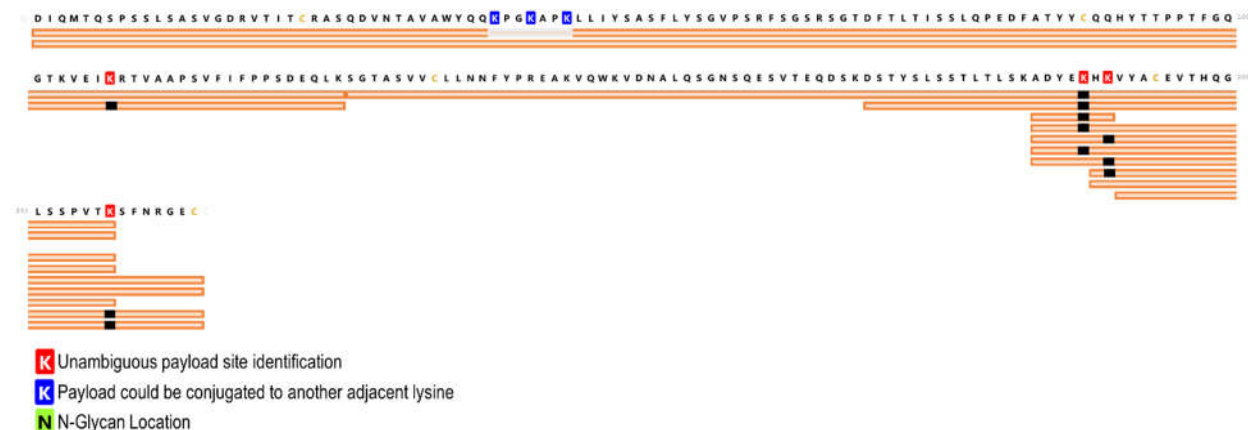


Figure S10. Peptide map displaying the global sequence coverage for the light chain. The same legend used in Figure 5 was retained for the heavy chain sequence. Peptides displayed in

orange contain a single payload conjugation. Unambiguously localized payload conjugations sites are shown in black on for each peptide, and payloads that could be localized two multiple sites are shown in gray.

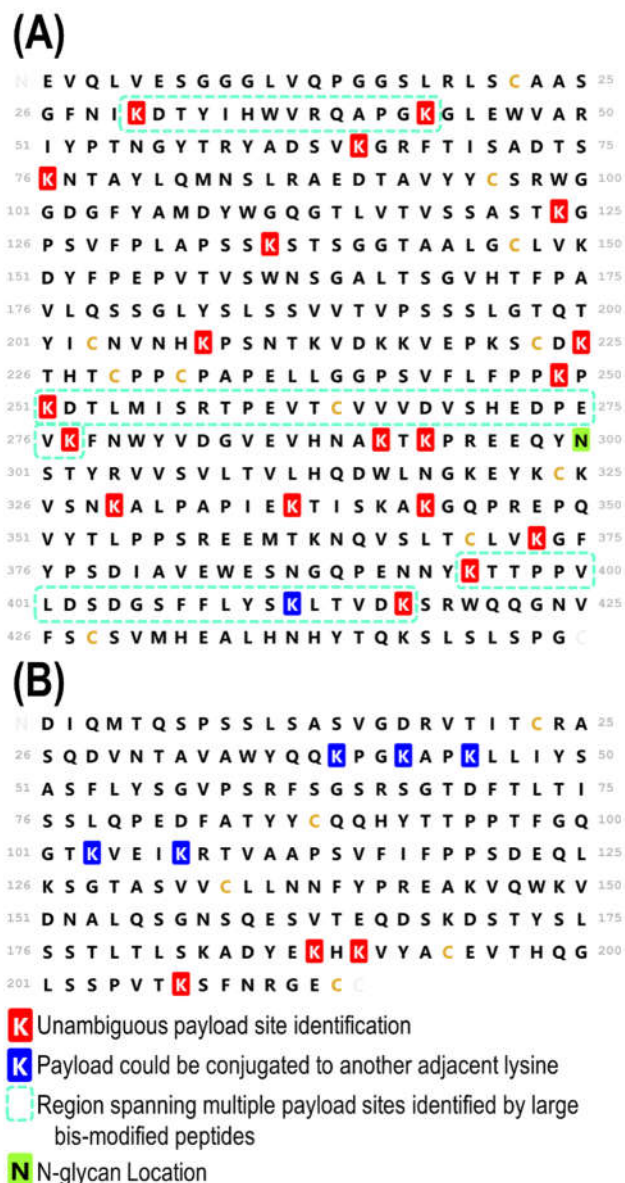


Figure S11. Sequence maps of the (A) heavy chain and (B) light chain of T-DM1 displaying the locations of the payloads, including those that were unambiguously localized (shared is red) and those that remain ambiguous with adjacent or nearby lysine residues (shaded in blue). Visualization displays results for secondary T-DM1 sample. Regions containing multiple payload sites that are identified by large bis-conjugated peptides are outlined in dashed boxes.

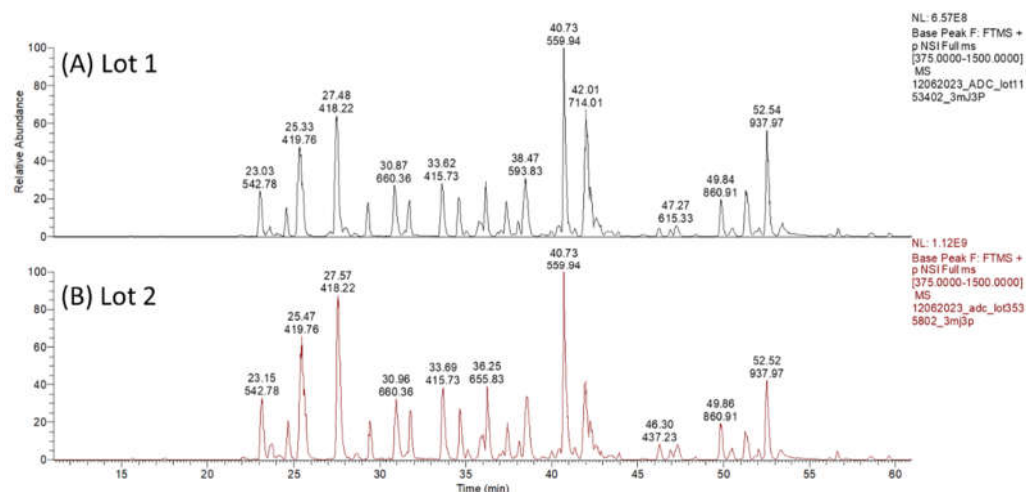


Figure S12. Base peak chromatograms of T-DM1 from two batches ((A) is Lot 1 and (B) is Lot 2) subjected to reduction, alkylation, and trypsin digestion separated using a 62 min gradient. Peaks are labeled with base peak m/z and retention times and represent unmodified peptides. Payload-containing peptides are at least 2 orders of magnitude lower and are not labeled.

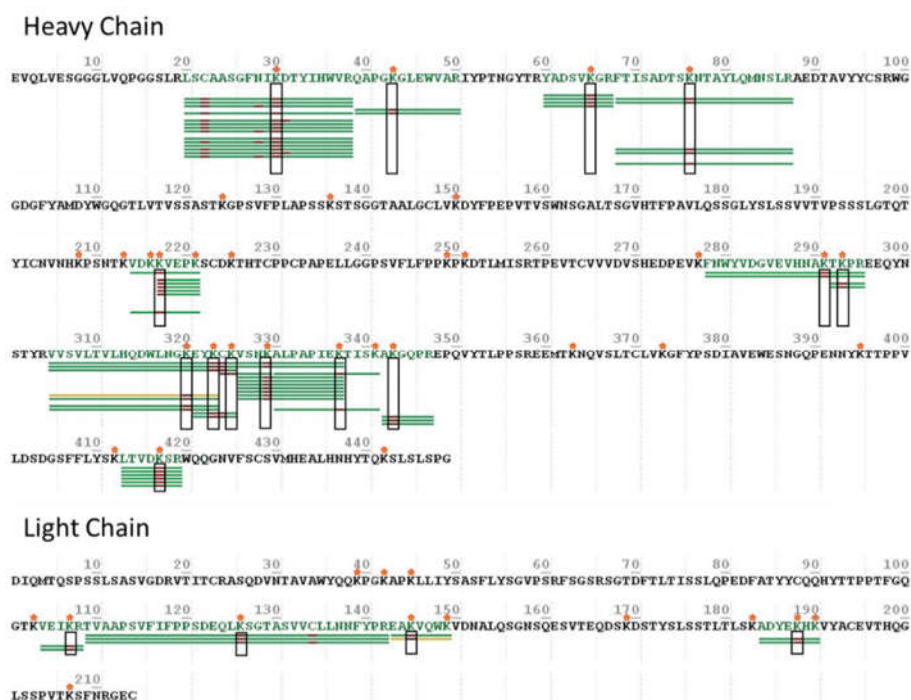
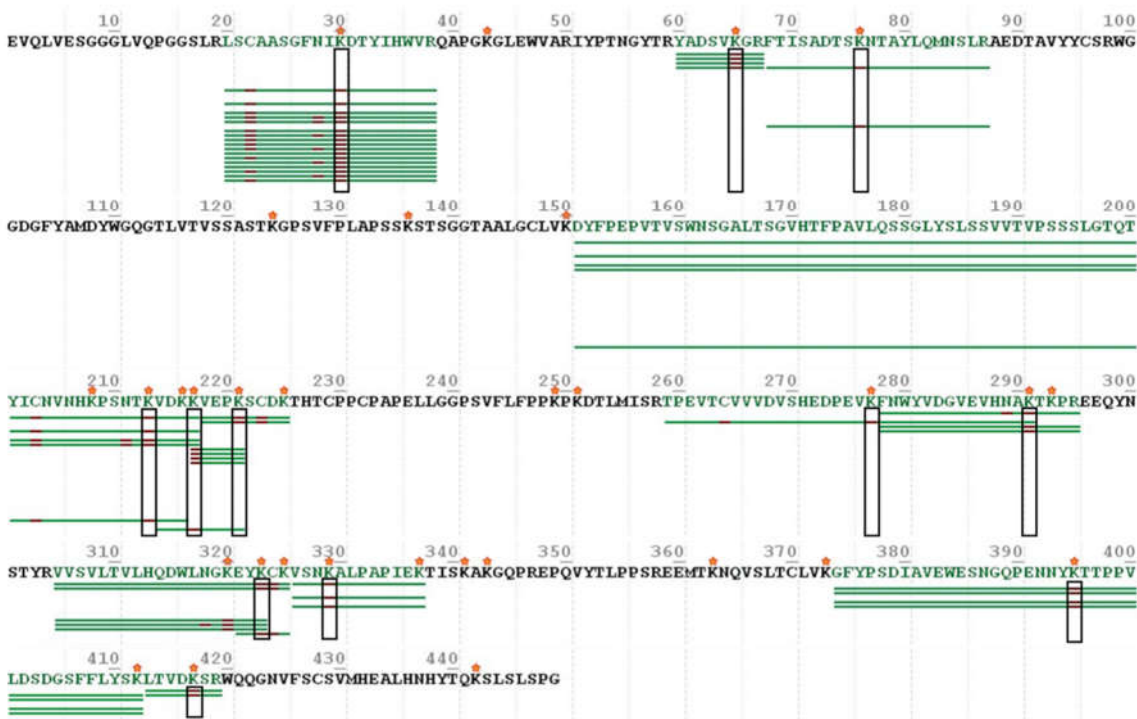


Figure S13: Sequence coverage maps of the heavy chain and light chain of TDM-1 based on LC-MS/MS (UVPD) analysis of a tryptic digest. Only payload-containing peptides

are shown in the maps, and these peptides are indicated by horizontal green bars. All lysines are labelled with red stars. All payload-modified lysines are outlined with black boxes. Fourteen payload-modified lysines were identified for the heavy chain, and four payload-modified peptides were identified for the light chain.

Heavy Chain



Light Chain



Figure S14. Sequence coverage maps of the heavy chain and light chain of TDM-1 based on LC-MS/MS (ET_hcD) analysis of a tryptic digest. Only payload-containing peptides are shown in the maps, and these peptides are indicated by horizontal green bars. All lysines are labelled with red stars. All payload-modified lysines are outlined with black boxes. Twelve payload-modified lysines were identified for the heavy chain, and five payload-modified peptides were identified for the light chain.

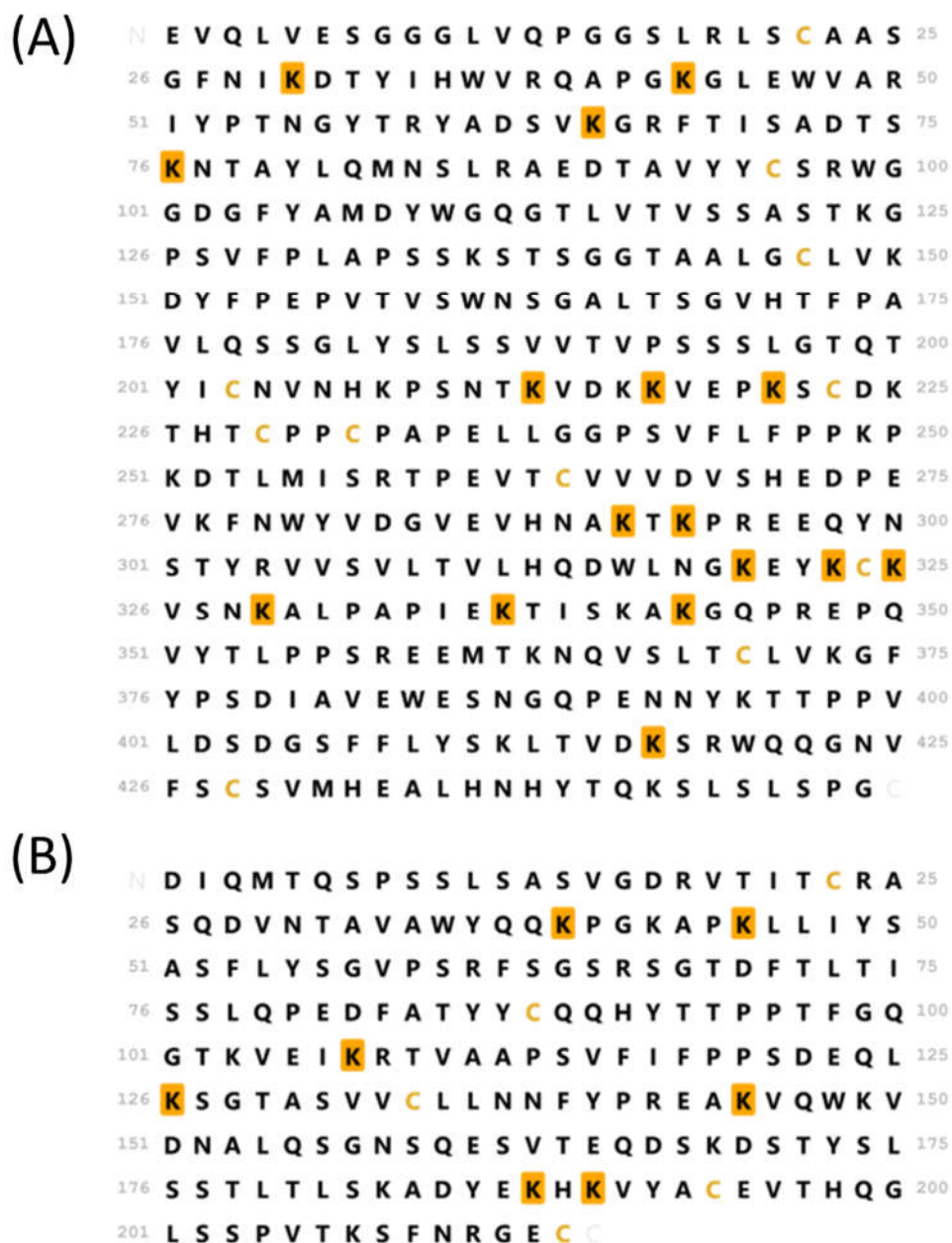


Figure S15. Sequence maps of the (A) heavy chain and (B) light chain of T-DM1 displaying the locations of the payloads identified using bottom-up analysis. Results from both batches of ADCs using both HCD-triggered EThcD and UVPD are combined. Modified lysines are shaded in gold.

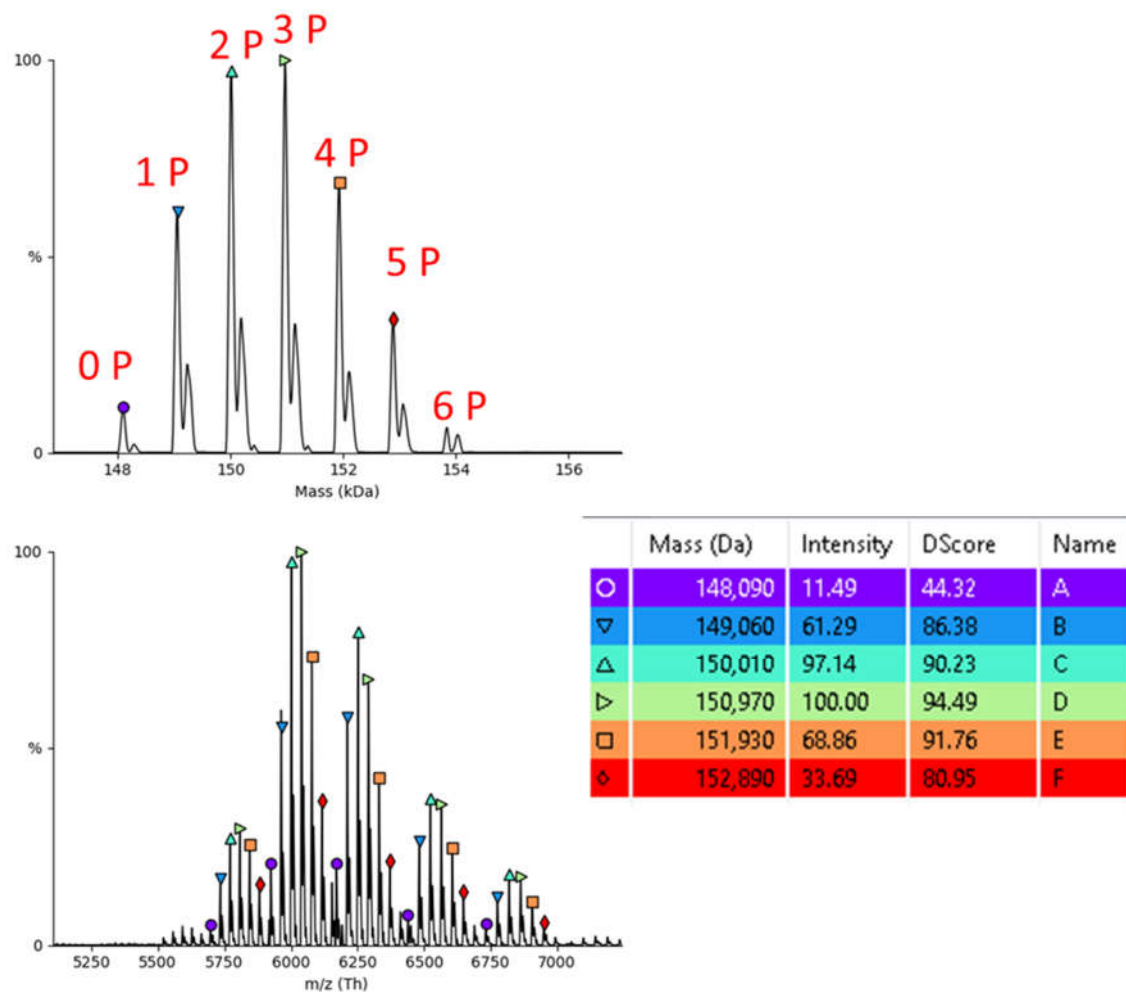
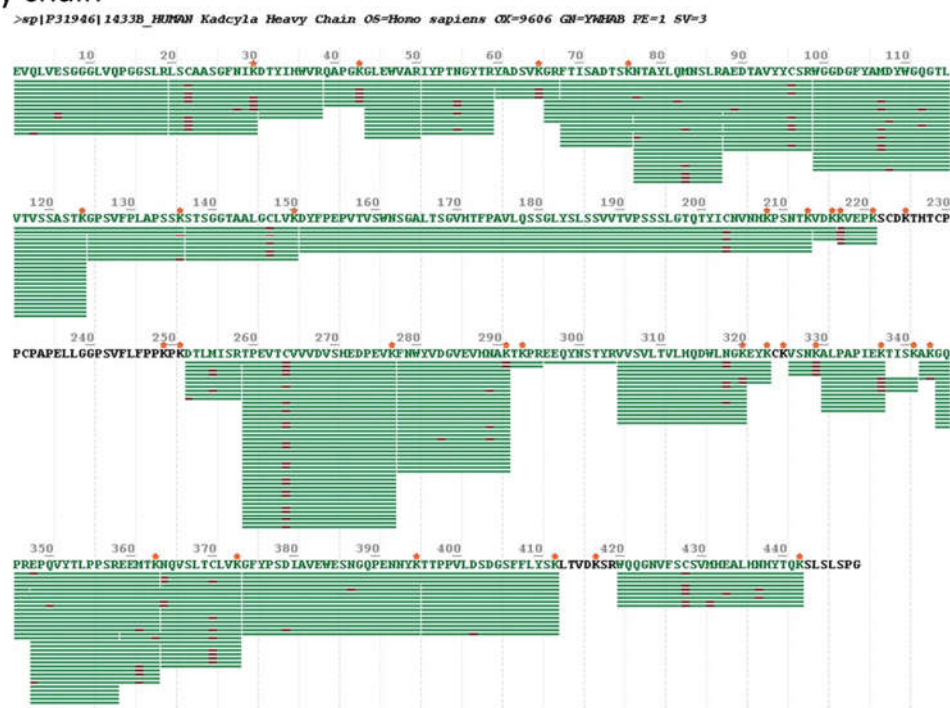


Figure S16. Mass spectrum of intact TDM-1 (lower) and deconvoluted mass spectrum (upper). The peaks are labelled with the number of attached payloads (P) in the deconvoluted mass spectrum.

(A) Heavy chain



(B) Light chain

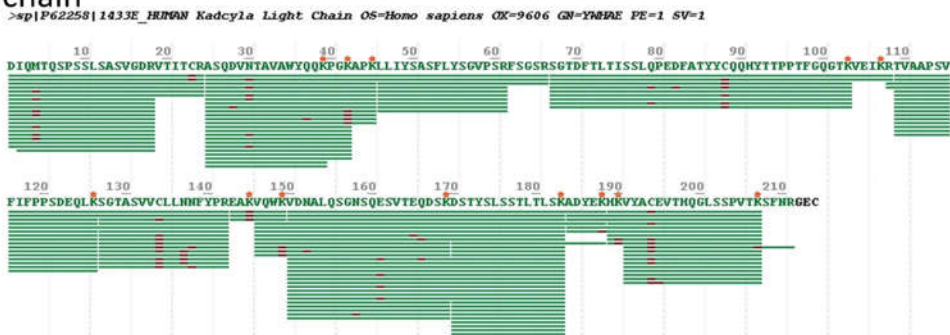


Figure S17. Peptide maps of the (A) heavy chain and (B) light chain of TDM-1 based on all identified peptides from the tryptic digest. All lysines are labelled with red stars. Lysines that are found to contain payloads are underlined with red dashes in the peptide sequences, all of which are marked with horizontal green bars.

Table S1. List of single payload-containing peptides identified with ProSight PD for UVPD replicates in primary sample. For each peptide, the residue to which the payload was localized, the theoretical mass, as well as the sequence coverage and retention time for each replicate are listed. Some replicate entries are blank in the case that a peptide was not identified in all five technical replicates.

Annotated Sequence	Payload Localization (residue number on peptide sequence)	Payload Localization (residue number on protein sequence)	Theo. Mass [Da]	Replicate 12		Replicate 3		Replicate 4		Replicate 5		
				SC	RT	SC	RT	SC	RT	SC	RT	
ADYEKHK	K5	LC-K188	1845.79	83%	26.1	83%	25.9	83%	26.0	83%	25.9	100% 26.2
ADYEKHKVYACEVTHQGLSSPVTK	K5	LC-K188	3645.68	96%	25.1	100%	24.9	96%	25.0	100%	24.9	96% 25.1
ADYEKHKVYACEVTHQGLSSPVTK	K7	LC-K190	3645.68	74%	25.8	70%	25.5			78%	25.6	52% 25.8
ADYEKHKVYACEVTHQGLSSPVTKSFNRGEC	K5	LC-K188	4439.00	90%	24.9	90%	24.8	93%	24.9	90%	24.8	90% 25.0
ADYEKHKVYACEVTHQGLSSPVTKSFNRGEC	K7	LC-K190	4439.00	67%	25.7	80%	25.5	73%	25.7	63%	25.4	63% 25.7
AKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNNHYTQK	K2	HC-K343	12487.96	56%	30.2	58%	30.2	43%	30.4	28%	29.9	35% 30.5
AKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNNHYTQK	K54	HC-K395	12487.96	71%	31.7	60%	31.4	68%	31.5	67%	31.4	77% 31.7
AKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNNHYTQK	K76	HC-K417	12487.96	58%	32.8	73%	32.4	67%	32.6	66%	32.5	67% 32.9
AKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNNHYTQKSLSLSPG	K2	HC-K343	13129.29	37%	30.4	28%	30.2	38%	30.4	52%	30.3	33% 30.7
AKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNNHYTQKSLSLSPG	K54	HC-K395	13129.29	65%	32.0	75%	31.6	62%	31.9	74%	31.6	74% 31.8
AKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNNHYTQKSLSLSPG	K71 or K76	HC-K412 or HC-K417	13129.29	61%	33.1	67%	32.9	63%	33.1	66%	33.0	59% 33.3
DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTVAAPSVFIFPPSDEQLK	K103 or K107	LC-K103 or LC-K107	14728.24	23%	33.3	30%	33.0	30%	33.0	22%	33.2	23% 33.4
DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTVAAPSVFIFPPSDEQLK	K39, K42, or K45	LC-K39, LC-K42, or LC-K45	14728.24	39%	34.2	36%	33.8	35%	33.9	35%	33.8	39% 34.0
DSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTK	K19	LC-K188	5129.42	54%	28.1	32%	27.8	43%	28.0	35%	27.9	41% 28.2
DTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAK	K26	HC-K277	5512.56	62%	35.6	67%	35.4	74%	35.5	56%	35.5	69% 35.6
DTYIHWVRQAPGKGLEWVARIYPTNGYTRYADSVK	K13	HC-K43	5066.45	29%	31.5	44%	31.2			24%	31.2	50% 31.5
DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVTPSSSLGTQTYICNVNHKPSNTK	K58	HC-K208	7611.65	40%	36.0	34%	35.9	32%	36.3	39%	35.9	23% 36.1
DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVTPSSSLGTQTYICNVNHKPSNTKVDK	K63	HC-K213	7953.84	46%	34.5	45%	34.3	57%	35.2	48%	34.4	65% 34.6
DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVTPSSSLGTQTYICNVNHKPSNTKVDKKVEPK	K58 or K63	HC-K208, or HC-K213	8535.19			66%	32.8	46%	33.1	44%	32.9	44% 33.1

DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPS NTKVDKKVEPK	K63, K66, or K67	HC-K213, HC- K216, or HC-K217	8535.19	36%	33.0	26%	31.7	51%	31.7	24%	31.5	34%	33.2
EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGK	K30	HC-K30	5480.70	74%	34.3	74%	34.1	69%	34.2	79%	34.1	71%	34.4
EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYT RYADSVK	K30	HC-K30	8020.98	72%	34.6	72%	34.4	75%	34.5	59%	34.5	64%	34.6
EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYT RYADSVK	K43	HC-K43	8020.98	45%	33.0	58%	32.7	56%	32.9	25%	32.8	52%	33.0
EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYT RYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT LVTVSSASTKGPSVFPLAPSSK	K30, or K43	HC-K30 or HC-K43	15667.61	25%	35.8	20%	35.7	33%	35.7	36%	35.7	32%	35.8
EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYT RYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT LVTVSSASTKGPSVFPLAPSSK	K65	HC-K65	15667.61	53%	34.9	43%	34.6	50%	34.9	45%	34.8	50%	34.9
EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYT RYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT LVTVSSASTKGPSVFPLAPSSK	K76	HC-K76	15667.61	53%	34.9	46%	35.0	47%	34.8	39%	34.8	44%	34.9
EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYT RYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT LVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVK	K65 or K76	HC-K65 or HC-K76	16913.25	11%	35.0	40%	34.6	30%	34.7	24%	34.7	25%	34.7
GLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWG GDGFYAMDYWGQGT	K22	HC-K65	11161.28	42%	36.3	51%	36.1	64%	36.2	68%	36.2	62%	36.3
GLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWG GDGFYAMDYWGQGT	K33	HC-K76	11161.28	48%	36.3	50%	36.1	64%	36.2	71%	36.2	58%	36.2
GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVL DSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQK	K52	HC-K395	12288.82	58%	32.4			69%	32.4	60%	32.3	59%	32.4
GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVL DSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQK	K69 or K74	HC-K412 or HC- K417	12288.82	43%	33.4	54%	33.3	45%	33.5	47%	33.2	56%	33.5
GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVL DSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG	K52	HC-K395	12930.16	62%	32.6			49%	32.7	34%	32.8	11%	33.8
GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVL DSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG	K69 or K74	HC-K412 or HC- K417	12930.16	55%	33.7	64%	33.6	39%	33.6	61%	33.5	65%	33.8
GRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT	K11	HC-K76	8621.00	73%	37.5	67%	37.3	69%	37.3	76%	37.4	61%	37.2
GRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT	K59	HC-K124	9866.64	46%	36.9			56%	36.9	33%	36.9	30%	37.0
HKVYACEVTHQGLSPPVTK	K2	LC-K190	3039.42	89%	26.3	72%	26.1	89%	26.2	83%	26.0	72%	26.3
HKVYACEVTHQGLSPPVTKSFNRGEC	K19	LC-K207	3832.73	44%	26.7	52%	26.5	52%	26.6	52%	26.4	60%	26.7
LTVDKSRWQQGNVFSCSVMHEALHNHYTQK	K5	HC-K417	4499.06	93%	28.6	93%	28.4	90%	28.5	90%	28.5	86%	28.7
NTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT	K48	HC-K124	7457.41	37%	38.9	27%	38.8	22%	39.3	46%	38.9	46%	39.0
NTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT	K60	HC-K136	8703.05	38%	38.2					38%	38.1	38%	38.3
SCDKTHTCPPCPAPELLGGPSVFLFPPKPK	K4	HC-K225	4118.93	79%	34.1	59%	34.7	66%	34.1	62%	34.0	86%	34.2

STSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPK	K80, K81, or K85	HC-K216, HC-K217, or HC-K221	9780.83	38%	33.8	30%	33.6	21%	33.7	44%	33.7			
THTCPPCPAPELLGGPSVFLFPPKPK	K24	HC-K249	3685.77	88%	37.9	84%	37.7	72%	37.9	76%	37.7	72%	37.9	
THTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVK	K26	HC-K251	6565.18	69%	35.4	51%	35.3	57%	35.3	55%	35.3	61%	35.4	
THTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK	K26	HC-K249	8223.96	43%	35.4	39%	35.2	49%	35.4	29%	35.3	66%	35.4	
THTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK	K52	HC-K277	8223.96	55%	35.3	49%	35.2	37%	35.3	40%	35.4	54%	35.4	
VSNKALPAIEK	K4	HC-K329	2222.10	82%	30.0	91%	29.8	100%	29.9	91%	29.8	91%	30.1	
TKPREEQYNSTYRVVSVLTVLHQDWLNGK	K2 and G0F N-Glycan	HC-K293 and G0F N-Glycan	5860.69	68%	31.5	61%	32.1	71%	31.4	54%	32.2	71%	31.5	
TKPREEQYNSTYRVVSVLTVLHQDWLNGK	K2 and G1F N-Glycan	HC-K293 and G1F N-Glycan	6022.74	68%	31.4	64%	31.1	68%	31.7	82%	31.2	71%	31.4	
FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK	K14 or K16 and G0F N-Glycan	HC-K291 or HC-K293 and G0F N-Glycan	7519.47	62%	31.5	76%	31.3	79%	31.4	41%	31.4	64%	31.6	
FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK	K14 or K16 and G1F N-Glycan	HC-K291 or HC-K293 and G0F N-Glycan	7681.52	69%	31.5	45%	31.2	74%	31.3	71%	31.3	64%	31.6	

Table S2. List of single payload-containing peptides identified with ProSight PD for EThcD replicates in primary sample. For each peptide, the residue to which the payload was localized, the theoretical mass, as well as the sequence coverage and retention time for each replicate are listed. Some replicate entries are blank in the case that a peptide was not identified in all five technical replicates.

Annotated Sequence	Payload Localization (residue number on peptide sequence)	Payload Localization (residue number on protein sequence)	Theo. Mass [Da]	Replicate 12		Replicate 3		Replicate 4		Replicate 5	
				SC	RT	SC	RT	SC	RT	SC	RT
ADYEKHK	K5	LC-K188	1845.79	100%	25.9	83%	26.1	100%	26.1	83%	26.1
ADYEKHKVYACEVTHQGLSSPVTK	K5	LC-K188	3645.68	83%	24.9	91%	25.1	83%	25.1	78%	25.0
ADYEKHKVYACEVTHQGLSSPVTK	K7	LC-K190	3645.68	74%	25.0	57%	25.8	83%	25.8	74%	25.1
ADYEKHKVYACEVTHQGLSSPVTKSFNRGEC	K5	LC-K188	4439.00	77%	24.8	77%	25.0	80%	25.0	80%	24.9
ADYEKHKVYACEVTHQGLSSPVTKSFNRGEC	K7	LC-K190	4439.00	57%	25.5	63%	25.7	63%	25.0	67%	25.5
AKGQPREPQVYTLPPSREEMTK	K2	HC-K343	3497.67	62%	25.9	38%	26.2	57%	26.1	29%	26.1
AKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCSCVMHEALHNNHYTQK	K2	HC-K343	12487.96			14%	30.6	29%	30.4	13%	30.4
AKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCSCVMHEALHNNHYTQK	K54	HC-K395	12487.96	42%	31.5	29%	31.7	50%	31.6	42%	31.5
AKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCSCVMHEALHNNHYTQK	K71 or K76	HC-K412 or HC-K417	12487.96	47%	32.7	45%	32.8	45%	32.7	46%	32.6
AKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCSCVMHEALHNNHYTQKSLSLSPG	K2	HC-K343	13129.29	21%	30.3	26%	30.5	25%	30.5	29%	30.4
AKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCSCVMHEALHNNHYTQKSLSLSPG	K54	HC-K395	13129.29	32%	31.9	31%	32.0	39%	31.8	33%	31.6
AKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCSCVMHEALHNNHYTQKSLSLSPG	K71 or K76	HC-K412 or HC-K417	13129.29	43%	33.1	41%	33.1	41%	33.2	36%	33.3
ALPAPIEKTISK	K8	HC-K337	2223.12	64%	32.4	55%	32.0				64%
DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVP	K103	LC-K103	14728.24	21%	33.1	18%	33.5	21%	33.3	15%	33.3
SRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTVAAPSVFIFPPSDEQLK											
DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVP	K39, K42, or K45	LC-K39, LC-K42, or LC-K45	14728.24	28%	34.1	28%	34.1	24%	33.6	23%	34.1
SRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTVAAPSVFIFPPSDEQLK											
DSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTK	K19	LC-K188	5129.42	41%	27.9	65%	28.1	62%	28.1	57%	28.0
DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK	K26	HC-K277	5512.56	51%	35.6	62%	35.8	49%	35.7	44%	35.6
DTYIHWVRQAPGKGLEWVARIYPTNGYTRYADSVK	K13	HC-K43	5066.45	24%	31.4	41%	31.6			35%	31.3
DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVTPSSSLGTQTYICNVNHKPSNTKVDK	K58	HC-K208	7953.84	20%	34.7	34%	34.7	23%	34.6	25%	34.5
DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVTPSSSLGTQTYICNVNHKPSNTKVDKKVEPK	K58 or K63	HC-K208, or HC-K213	8535.19	46%	33.1	41%	33.2	59%	33.1	31%	33.1

DYFPEPVTVSWNSGALTSVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPS NTKVDKKVEPK	K63, K66, or K67	HC-K213, HC- K216, or HC-K217	8535.19	46%	31.6	59%	31.8	30%	31.7	29%	31.6	33%	31.6
EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGK	K30	HC-K30	5480.70	71%	34.2	76%	34.4	69%	34.5	67%	34.3	74%	34.2
EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYT RYADSVK	K30	HC-K30	8020.98	55%	34.5	59%	34.6	47%	34.7	64%	34.6	59%	34.6
EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYT RYADSVK	K43	HC-K43	8020.98			48%	33.0	53%	33.0	42%	33.0	42%	32.9
EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYT RYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT LVTVSSASTKGPSVFPLAPSSK	K30	HC-K30	15667.61	19%	35.7	17%	35.9	21%	35.8	22%	35.8	21%	35.8
EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYT RYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT LVTVSSASTKGPSVFPLAPSSK	K65	HC-K65	15667.61	31%	34.7	30%	35.0	33%	34.8	29%	34.8	30%	34.9
EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYT RYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT LVTVSSASTKGPSVFPLAPSSK	K76	HC-K76	15667.61	30%	34.7	25%	35.0	33%	34.8	22%	34.8	23%	34.7
EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYT RYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT LVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVK	K65 or K76	HC-K65 or HC-K76	16913.25	9%	34.7	24%	34.8	13%	34.8	26%	34.7		
GLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWG DGFYAMDYWGQGT LVTVSSASTKGPSVFPLAPSSK	K22	HC-K65	11161.28	34%	36.2	42%	36.3	58%	36.2	55%	36.3	47%	36.2
GLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWG DGFYAMDYWGQGT LVTVSSASTKGPSVFPLAPSSK	K33	HC-K76	11161.28			37%	36.4			58%	36.3	37%	36.2
GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVL DSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQK	K52	HC-K395	12288.82	26%	32.6	48%	32.6	36%	32.6	34%	32.3	34%	32.7
GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVL DSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQK	K69 or K74	HC-K412 or HC- K417	12288.82	39%	33.4	41%	33.5	31%	33.4	30%	33.5	33%	33.5
GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVL DSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSPG	K69 or K74	HC-K412 or HC- K417	12930.16	33%	33.6	37%	33.9	31%	33.8	22%	33.8	31%	33.6
GRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT LVTVSSAS TKGPSVFPLAPSSK	K11	HC-K76	8621.00	83%	37.2	86%	37.1	79%	37.3	87%	37.1	81%	37.3
GRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT LVTVSSAS TKGPSVFPLAPSSKSTSGGTAALGCLVK	K59	HC-K124	9866.64	44%	36.8	52%	37.0			23%	37.3	37%	36.9
HKVYACEVTHQGLSSPVTK	K2	LC-K190	3039.42	78%	26.0	83%	26.3	89%	26.2	78%	26.1	78%	26.1
HKVYACEVTHQGLSSPVTKSFNRGEC	K19	LC-K207	3832.73	40%	26.4	36%	26.7	40%	26.7	56%	26.5		
LTVDKSRWQQGNVFSCSVMHEALHNHYTQK	K5	HC-K417	4499.06	93%	28.4	86%	28.7	90%	28.6	97%	28.5	90%	28.5
NTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT LVTVSSASTKGPSVFPLA PSSK	K48	HC-K124	7457.41	46%	39.0	61%	39.1	53%	39.4	49%	39.0	58%	38.9
NTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT LVTVSSASTKGPSVFPLA PSSKSTSGGTAALGCLVK	K60	HC-K136	8703.05	26%	38.1	22%	38.3	11%	38.3	15%	38.5	26%	38.1
SCDKTHTCPPCPAPELLGGPSVFLFPPKPK	K4	HC-K225	4118.93	79%	34.2	83%	34.3	90%	34.2	83%	34.1	90%	34.1
SGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSYSLSTLTLSKA DYEKHKVYACEVTHQGLSSPVTK	K62	LC-K188	9837.70	38%	28.4	43%	28.6	25%	28.5	26%	28.5	40%	28.4
THTCPPCPAPELLGGPSVFLFPPKPK	K24	HC-K249	3685.77	72%	37.7	64%	38.0	64%	38.0	72%	38.0	68%	38.0

THTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVK	K24 or K26	HC-K249 or HC-K251	6565.18	43%	35.2	49%	35.4	41%	35.5	37%	35.4	39%	35.4
THTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK	K24 or K26	HC-K249 or HC-K251	8223.96	23%	35.5			32%	35.6	26%	35.6	28%	35.5
THTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK	K52	HC-K277	8223.96	46%	35.2	43%	35.6	39%	35.4	26%	35.5	45%	35.3
VSNKALPAIEK	K4	HC-K329	2222.10	91%	29.8	82%	30.1	73%	30.0	91%	29.9	91%	29.9
VYACEVTHQGLSSPVTKSFNRGEC	K17	LC-K207	3567.58	30%	29.8	26%	30.1	26%	30.0	35%	29.9		
TKPREEQYNSTYRVVSVLTVLHQDWLNGK	K2 and G0F N-Glycan	HC-K293 and G0F N-Glycan	5860.69	82%	31.2	82%	31.9	82%	31.8	82%	31.4	64%	31.4
TKPREEQYNSTYRVVSVLTVLHQDWLNGK	K2 and G1F N-Glycan	HC-K293 and G1F N-Glycan	6022.74	71%	31.7	75%	31.4	79%	31.3	75%	31.2	71%	31.3
FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK	K14 and G0F N-Glycan	HC-K291 and G0F N-Glycan	7519.47	71%	31.4	79%	31.6	76%	31.6	64%	31.4	81%	31.4
FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK	K14 and G1F N-Glycan	HC-K291 and G0F N-Glycan	7681.52	64%	31.3	79%	31.5	62%	31.6	71%	31.4	67%	31.4

Table S4. List of two payload-containing peptides identified with ProSight PD for UVPD replicates in primary sample. For each peptide, the residue to which the payload was localized, the theoretical mass, as well as the sequence coverage and retention time for each replicate are listed. Some replicate entries are blank in the case that a peptide was not identified in all five technical replicates.

Annotated Sequence	Payload Localization (residue number on peptide sequence)	Payload Localization (residue number on protein sequence)	Theo. Mass [Da]	Replicate 1		Replicate 2		Replicate 3		Replicate 4		Replicate 5	
				SC	RT	SC	RT	SC	RT	SC	RT	SC	RT
AKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYkTTPPVLDSDGSFFLYSKLTVDkSRWQQGNVFSCSVMHEALHNHYTQK	K54 and K71 or K76	HC-K395 and HC-K412 or HC-K417	13444.32	76%	38.5	78%	38.3	63%	38.3	59%	38.6	40%	38.5
EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGkGLEWVARIYPTNGYT RYADSVK	K30 and K43	HC-K30 and HC-K43	8977.34	53%	41.8	31%	41.6	16%	41.7	38%	41.7	42%	41.9
GFYPSDIAVEWESNGQPENNYkTTPPVLDSDGSFFLYSKLTVDkSRWQQGNVFSCSVM HEALHNHYTQK	K22 and K44	HC-K395 and HC-K417	9835.44	65%	40.6	40%	40.5	72%	40.6	50%	40.6	69%	40.9
GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYkTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQK	K52 and K69 or K74	HC-K395 and HC-K412 or HC-K417	13245.19	17%	39.0					35%	38.8	63%	38.8
NQVSLTCLVKGFYPSDIAVEWESNGQPENNYkTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQK	K32 and K49 or K54	HC-K395 and HC-K412 or HC-K417	10921.03	63%	40.5	35%	40.4	56%	40.5	39%	40.5	51%	40.7
THTCPPCPAPELLGGPSVFLFPPKPkDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAK	K26 and K52	HC-K251 and HC-K277	9180.32	25%	41.8	34%	41.8	46%	41.8	28%	41.9	15%	42.0

Table S5. List of two payload-containing peptides identified with ProSight PD for EThcD replicates in primary sample. For each peptide, the residue to which the payload was localized, the theoretical mass, as well as the sequence coverage and retention time for each replicate are listed. Some replicate entries are blank in the case that a peptide was not identified in all five technical replicates.

Annotated Sequence	Payload Localization (residue number on peptide sequence)	Payload Localization (residue number on protein sequence)	Theo. Mass [Da]	Replicate 1		Replicate 2		Replicate 3		Replicate 4		Replicate 5	
				SC	RT	SC	RT	SC	RT	SC	RT	SC	RT
ADYEKHKVYACEVTHQGLSSPVTK	K5 and K7	LC-K188 and LC-K190	4602.05	30%	37.3			35%	38.0	35%	37.3		
AKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYkTTPPVLDSDGSFFLYSKLTVDkSRWQQGNVFSCSVMHEALHNHYTQK	K54 and K71 or K76	HC-K395 and HC-K412 or HC-K417	13444.32	38%	38.3	35%	38.0	35%	38.5	38%	38.1	42%	38.3
EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGkGLEWVARIYPTNGYT RYADSVK	K30 and K43	HC-K30 and HC-K43	8977.34			23%	41.9	20%	41.8	25%	41.9		
GFYPSDIAVEWESNGQPENNYkTTPPVLDSDGSFFLYSKLTVDkSRWQQGNVFSCSVMHEALHNHYTQK	K22 and K44	HC-K395 and HC-K417	9835.44	54%	40.6	56%	40.6	52%	40.6	49%	40.5	56%	40.6
GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYkTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQK	K52 and K69 or K74	HC-K395 and HC-K412 or HC-K417	13245.19					28%	38.8	34%	38.9	16%	38.8
NQVSLTCLVKGFYPSDIAVEWESNGQPENNYkTTPPVLDSDGSFFLYSKLTVDkSRWQQGNVFSCSVMHEALHNHYTQK	K32 and K54	HC-K395 and HC-K412 or HC-K417	10921.03	36%	40.5	31%	40.6	45%	40.6	42%	40.5	35%	40.5
THTCPPCPAPELLGGPSVFLFPPkPKDTLMISRTPEVTCVVDVSHEDPEVkFNWYVDGVEVHNAK	K24 or K26 and K52	HC-K249 or HC-K251 and HC-K277	9180.32	34%	41.9	34%	42.0	29%	42.0	31%	41.8	26%	41.8

Table S6. List of single payload-containing peptides identified with ProSight PD for UVPD replicates in secondary sample. For each peptide, the residue to which the payload was localized, the theoretical mass, as well as the sequence coverage and retention time for each replicate are listed. Some replicate entries are blank in the case that a peptide was not identified in all five technical replicates.

Annotated Sequence	Payload Localization (residue number on peptide sequence)	Payload Localization (residue number on protein sequence)	Theo. Mass [Da]	Replicate 12		Replicate 3		Replicate 4		Replicate 5			
				SC	RT	SC	RT	SC	RT	SC	RT		
ADYEKHK	K5	LC-K188	1845.79	83%	25.6	67%	25.5	83%	25.8	100%	25.8	100%	26.1
ADYEKHKVYACEVTHQGLSSPVTK	K5	LC-K188	3645.68	83%	25.0	70%	24.9	87%	25.1	83%	24.9	87%	25.2
ADYEKHKVYACEVTHQGLSSPVTKSFNRGEC	K5	LC-K188	4439.00	83%	24.7	83%	24.8	80%	24.9	80%	24.8	80%	25.2
AKGQPREPQVYTLPPSREEMTK	K2	HC-K343	3497.66	67%	25.6	67%	25.5	71%	25.7	57%	25.8	76%	26.1
AKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMEALHNHYTQK	K32	HC-K373	12487.96	36%	31.4	22%	29.8	24%	31.3	39%	31.4	30%	31.6
AKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMEALHNHYTQK	K54	HC-K395	12487.96	39%	31.3	43%	31.1	55%	31.3	48%	31.3	40%	31.6
AKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMEALHNHYTQK	K54, K71 or K76	HC-K395, HC-K412, or HC-K417	12487.96	47%	32.5	41%	32.4	34%	32.4	41%	32.5	42%	32.7
AKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMEALHNHYTQKSLSLSPG	K71 or K76	HC-K412 or HC-K417	13129.29	35%	32.9	36%	32.7	36%	32.9	35%	33.0	38%	33.1
DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTVAAPSVFIFPPSDEQLK	K103 or K107	LC-K103 or LC-K107	14728.24	15%	33.0	17%	32.8	17%	32.9			18%	33.2
DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTVAAPSVFIFPPSDEQLK	K39, K42, or K45	LC-K39, LC-K42, or LC-K45	14728.24	22%	34.1	24%	34.1	19%	33.8	20%	33.8	22%	34.2
DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK	K26	HC-K277	5512.56	74%	35.4	56%	35.3	54%	35.5	59%	35.5	62%	35.5
DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTK	K58	HC-K208	7611.65	21%	36.3	21%	35.7					19%	35.9
EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT LVTVSSASTKGPSVFPLAPSSK	K65 or K76	HC-K65 or HC-K76	15667.61	21%	34.8	14%	34.6			24%	34.8	22%	34.9
EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT LVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVK	K65 or K76	HC-K65 or HC-K76	16913.25	22%	34.7			19%	34.7	24%	34.7	19%	34.8
EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRYADSVK	K30 or K43	HC-K30 or HC-K43	8020.98	16%	32.7	20%	34.4	25%	34.5				
EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGK	K30	HC-K30	5480.70	60%	34.0	52%	34.1	52%	34.3	50%	34.1	55%	34.2
GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMEALHNHYTQK	K39 or K44	HC-K412 or HC-K417	8879.08	43%	34.2			27%	34.3	47%	34.2	27%	34.4

GLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWG GDGFYAMDYWGQGLTVTVSSASTKGPSVFPLAPSSK	K22	HC-K65	11161.28	37%	36.2	42%	36.0	24%	36.2	37%	36.1	40%	36.2
GLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWG GDGFYAMDYWGQGLTVTVSSASTKGPSVFPLAPSSK	K33	HC-K76	11161.28	37%	36.2	42%	36.0	35%	36.2	36%	36.1	39%	36.2
GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVL DSDGSFFLYSKLTVDKSRWQQGNVFCSCVMHEALHNHYTQK	K52	HC-395	12288.82	40%	32.3	29%	32.9	35%	32.2	34%	32.2	35%	32.4
GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVL DSDGSFFLYSKLTVDKSRWQQGNVFCSCVMHEALHNHYTQK	K69 or K74	HC-K412 or HC- K417	12288.82	36%	33.1	29%	32.9	31%	33.2	22%	33.3	31%	33.3
GRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWG GDGFYAMDYWGQGLTVTVSSASTKGPSVFPLAPSSK	K11	HC-K76	8621.00	56%	37.3	54%	37.0	50%	37.3	53%	37.4	56%	37.3
GRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWG GDGFYAMDYWGQGLTVTVSSASTKGPSVFPLAPSSK STSGGTAALGCLVK	K59 or K71	HC-K124 or HC- K136	9866.64	14%	36.8	38%	36.8	14%	36.8			18%	36.9
HKVYACEVTHQGLSSPVTK	K2	LC-K190	3039.42	67%	25.6	56%	25.7	67%	25.9	78%	25.9	61%	26.2
LTVDKSRWQQGNVFCSCVMHEALHNHYTQK	K5	HC-K417	4499.06	93%	28.2	97%	27.8	97%	28.2	93%	28.2	86%	28.8
NTAYLQMNSLRAEDTAVYYCSRWG GDGFYAMDYWGQGLTVTVSSASTKGPSVFPLA PSSK	K48	HC-K124	7457.41	32%	39.3	39%	38.8	44%	38.9	34%	39.0	42%	38.9
NTAYLQMNSLRAEDTAVYYCSRWG GDGFYAMDYWGQGLTVTVSSASTKGPSVFPLA PSSK STSGGTAALGCLVK	K60	HC-K136	8703.05	27%	38.2	27%	38.1	36%	38.1	36%	38.1	38%	38.1
SCDKTHTCPPCPAPELLGGPSVFLFPPKPK	K4	HC-K225	4118.93	69%	34.5	69%	34.0	69%	34.1	66%	33.9	66%	34.0
SGTASVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKSTYLSSTLTLSKA DYEKHKVYACEVTHQGLSSPVTKSFNRGEC	K57, K62, or K64	LC-K183, LC-K188, or LC-K19	10631.02	26%	27.7	28%	27.6	40%	27.9	32%	28.1	30%	28.3
THTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDG VEVHNAK	K24, or K26	HC-K249 or HC- K251	8223.96	34%	35.3	35%	35.1	29%	35.1	28%	35.1	25%	35.4
THTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDG VEVHNAK	K52	HC-K277	8223.96	26%	35.3	26%	35.0	17%	35.2	45%	35.0	37%	35.2
THTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVK	K26	HC-K251	6565.18	35%	35.4	45%	35.0	41%	35.1	53%	35.1	45%	35.2
THTCPPCPAPELLGGPSVFLFPPKPK	K24	HC-K249	3685.77	72%	37.7	60%	37.7	72%	37.7	60%	37.9	72%	37.8
VSNKALPAIEK	K4	HC-K329	2222.10	82%	29.4	82%	29.3	82%	29.6	64%	29.6	73%	30.0
VYACEVTHQGLSSPVTKSFNRGEC	K17	LC-K207	3567.58	44%	29.5	48%	29.4	39%	29.6	35%	29.7	39%	30.0
TKPREEQYNSTYRVVSVLTVLHQDWLNGK	K2 and G0F N- Glycan	HC-K293 and G0F N-Glycan	5860.69	54%	31.8	61%	30.6	54%	31.1	54%	31.8	50%	31.9
TKPREEQYNSTYRVVSVLTVLHQDWLNGK	K2 and G1F N- Glycan	HC-K293 and G1F N-Glycan	6022.74	50%	30.9	64%	30.7	57%	31.0	54%	31.0	54%	31.9
DTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSV LTVLHQDWLNGK	K40 or K42 and G0F N-Glycan	HC-K291 or HC- K293 and G0F N- Glycan	10398.88			28%	31.8	31%	31.6	34%	31.6	32%	31.7
FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK	K14 or K16 and G0F N-Glycan	HC-K291 or HC- K293 and G0F N- Glycan	7519.47	67%	31.1			45%	31.2	52%	31.2	48%	31.4
FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK	K14 or K16 and G1F N-Glycan	HC-K291 or HC- K293 and G1F N- Glycan	7681.52	38%	31.0	41%	30.8	31%	31.1	50%	31.2	41%	31.3

Table S7. List of single payload-containing peptides identified with ProSight PD for EThcD replicates in secondary sample. For each peptide, the residue to which the payload was localized, the theoretical mass, as well as the sequence coverage and retention time for each replicate are listed. Some replicate entries are blank in the case that a peptide was not identified in all five technical replicates.

Annotated Sequence	Payload Localization (residue number on peptide sequence)	Payload Localization (residue number on protein sequence)	Theo. Mass [Da]	Replicate 12		Replicate 3		Replicate 4		Replicate 5	
				SC	RT	SC	RT	SC	RT	SC	RT
ADYEKHK	K5	LC-K188	1845.79	100 %	25.9	83%	26.0	100 %	26.6	100 %	26.0
ADYEKHKVYACEVTHQGLSSPVTK	K5	LC-K188	3645.68	74%	24.9	87%	25.1	83%	25.1	96%	25.2
ADYEKHKVYACEVTHQGLSSPVTK	K7	LC-K190	3645.68	30%	25.0	35%	25.8			83%	25.7
ADYEKHKVYACEVTHQGLSSPVTKSFNRGEC	K5	LC-K188	4439.00	80%	24.7	80%	25.1	77%	24.8	77%	25.0
ADYEKHKVYACEVTHQGLSSPVTKSFNRGEC	K7	LC-K190	4439.00	57%	24.7	57%	25.6	73%	25.7	63%	25.6
AKGQPREPQVYTLPPSREEMTK	K2	HC-K343	3497.66	67%	25.8	48%	26.4	62%	26.1	67%	26.4
AKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP VLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQK	K2	HC-K343	12487.96	25%	30.2	8%	30.4			17%	30.2
AKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP VLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQK	K54	HC-K395	12487.96	46%	31.4	40%	31.6	40%	31.5	39%	31.7
AKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP VLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQK	K71 or K76	HC-K412 or HC-K417	12487.96	46%	32.5	47%	32.7	50%	32.6	47%	32.8
AKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP VLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPG	K54	HC-K395	13129.29	37%	31.6	38%	31.7			47%	31.7
AKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP VLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPG	K71 or K76	HC-K412 or HC-K417	13129.29	40%	33.0	44%	33.1	36%	33.1	41%	33.1
ALPAPIEKTISK	K8	HC-K337	2223.12	73%	31.6	73%	31.8	55%	31.6	73%	31.8
DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVP SRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTVAAPSVFIFPPS DEQLK	K38, K42, or K45	LC-K39, LC-K42, or LC-K45	14728.24	26%	33.8	22%	33.8	29%	33.8	26%	34.0
DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVP SRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTVAAPSVFIFPPS DEQLK	K103 or K107	LC-K103 or LC-K107	14728.24	16%	33.1	16%	33.0	20%	33.0	18%	33.3
DSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC	K19 or K21	LC-K188 or LC-K190	5922.74	43%	27.9	18%	28.1	66%	27.7	30%	28.2
DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK	K26	HC-K277	5512.56	54%	35.5	54%	35.5	39%	35.4	54%	35.6
DTYIHWVRQAPGKGLEWVARIYPTNGYTRYADSVK	K13	HC-K43	5066.45			62%	31.3	56%	31.3	59%	31.4
DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVTPSSSLGTQTYICNVNHKPS NTK	K58	HC-K208	7611.65	37%	35.8	29%	35.9	18%	35.9	23%	36.0
EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGK	K30	HC-K30	5480.70	67%	34.1	64%	34.2	67%	34.2	62%	34.3
EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYT RYADSVK	K30	HC-K30	8020.98	31%	34.4	23%	34.5	44%	34.5	50%	34.6

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYT RYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT LTVTVSSASTKGPSVFPLAPSSK	K65 or K76	HC-K65 or HC-K76	15667.61	22%	34.8	23%	34.8	13%	34.9	16%	35.0	23%	34.9
GFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSV MHEALHNHYTQK	K44	HC-K417	8879.08	59%	34.2	52%	34.3	44%	34.3	44%	34.4	46%	34.3
GLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRW GDGFYAMDYWGQGT LTVTVSSASTKGPSVFPLAPSSK	K22	HC-K65	11161.28	26%	36.1	47%	36.1	55%	36.2	45%	36.3	44%	36.2
GLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRW GDGFYAMDYWGQGT LTVTVSSASTKGPSVFPLAPSSK	K33	HC-K76	11161.28	26%	36.1	47%	36.1	41%	36.1			44%	36.2
GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPV LSDGSFFLYSKLTVDKSRWQQGNVFSCSV MHEALHNHYTQK	K52	HC-395	12288.82	36%	32.2	42%	32.5	32%	32.4	38%	32.6	34%	32.4
GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPV LSDGSFFLYSKLTVDKSRWQQGNVFSCSV MHEALHNHYTQK	K69 or K74	HC-K412 or HC- K417	12288.82	35%	33.2	21%	33.4	36%	33.2	33%	33.5	17%	33.6
GRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT LTVTVSSA TKGPSVFPLAPSSK	K11	HC-K76	8621.00	87%	37.3	80%	37.3	74%	37.3	69%	37.7	87%	37.2
HKVYACEVTHQGLSPVTK	K2	LC-K190	3039.42	83%	25.9	78%	26.2	83%	26.2	83%	26.2	89%	26.2
LTVDKSRWQQGNVFSCSV MHEALHNHYTQK	K5	HC-K417	4499.06	90%	28.3	93%	28.7	90%	28.7	90%	28.6	97%	28.6
NTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT LTVTVSSASTKGPSVFPLA PSSK	K48	HC-K124	7457.41	63%	38.9	44%	38.9	49%	38.8	56%	38.9	51%	38.8
NTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT LTVTVSSASTKGPSVFPLA PSSKSTSGGTAALGCLVK	K60	HC-K136	8703.05	22%	38.1	23%	38.1	21%	38.0	19%	38.2	19%	38.1
SCDKTHTCPPCPAPELLGGPSVFLFPPKPK	K4	HC-K225	4118.93	86%	33.9	83%	33.9	83%	34.0	76%	34.0	76%	34.1
SGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKSTYLSSTLTLSKA DYEKHKVYACEVTHQGLSPVTKSFNRGEC	K62	LC-K188	10631.02	31%	28.1	22%	28.4	26%	28.4	22%	28.4	36%	28.4
THTCPPCPAPELLGGPSVFLFPPKPK	K24	HC-K249	3685.77	68%	37.7	88%	37.8	68%	37.6	68%	37.8	68%	37.7
THTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVK	K24 or K26	HC-K249 or HC- K251	6565.18	53%	35.1	31%	35.5	49%	35.1	37%	35.3	33%	35.2
THTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDG VEVHNAK	K26	HC-K251	8223.96	31%	35.2	31%	35.4	15%	35.4	22%	35.4	31%	35.4
THTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDG VEVHNAK	K52	HC-K277	8223.96	45%	35.1	32%	35.3	40%	35.2	40%	35.3	32%	35.4
VSNKALPAPIEK	K4	HC-K329	2222.10	91%	29.8	100 %	29.9	73%	29.9	91%	30.0	82%	29.9
VYACEVTHQGLSPVTKSFNRGEC	K17	LC-K207	3567.58					35%	30.0	35%	30.0	26%	30.1
TKPREEQYNSTYRVVSVLTVLHQDWLNGK	K2 and G0F N- Glycan	HC-K293 and G0F N-Glycan	5860.69	79%	31.2	68%	31.9	75%	31.4	79%	31.4	61%	31.4
TKPREEQYNSTYRVVSVLTVLHQDWLNGK	K2 and G1F N- Glycan	HC-K293 and G1F N-Glycan	6022.74	86%	31.1	79%	31.3	75%	31.3	82%	31.4	71%	31.3
DTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSV LTVLHQDWLNGK	K40 and G0F N- Glycan	HC-K291 and G0F N-Glycan	10398.88	29%	31.6	47%	31.8	52%	31.7	41%	31.8	22%	31.7
FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK	K14 and G0F N- Glycan	HC-K291 and G0F N-Glycan	7519.47	86%	31.3	64%	31.5	74%	31.4	79%	31.5	55%	31.5
FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK	K14 and G1F N- Glycan	HC-K291 and G1F N-Glycan	7681.52	71%	31.3	71%	31.4	81%	31.3	81%	31.4	48%	31.4

Table S8. List of two payload-containing peptides identified with ProSight PD for UVPD replicates in secondary sample. For each peptide, the residue to which the payload was localized, the theoretical mass, as well as the sequence coverage and retention time for each replicate are listed. Some replicate entries are blank in the case that a peptide was not identified in all five technical replicates.

Annotated Sequence	Payload Localization (residue number on peptide sequence)	Payload Localization (residue number on protein sequence)	Theo. Mass [Da]	Replicate 1		Replicate 2		Replicate 3		Replicate 4		Replicate 5	
				SC	RT	SC	RT	SC	RT	SC	RT	SC	RT
AKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYkTTPPVLDSDGSFFLYskLTVDKSRWQQGNVFSCSVMHEALHNHYTQK	K54 and K71 or K76	HC-K395 and HC-K412 or HC-K417	13444.32	29%	38.5	35%	38.3	34%	38.3	45%	38.2	36%	38.3
GFYPSDIAVEWESNGQPENNYkTTPPVLDSDGSFFLYskLTVDKSRWQQGNVFSCSVMHEALHNHYTQK	K22 and K39 or K44	HC-K395 and HC-K412 or HC-K417	9835.44	37%	40.6	41%	40.5	34%	40.6	18%	40.5	21%	40.5
GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYkTTPPVLDSDGSFFLYskLTVDKSRWQQGNVFSCSVMHEALHNHYTQK	K52 and K69 or K74	HC-K395 and HC-K412 or HC-K417	13245.19	19%	38.9			25%	38.8			30%	38.8
NQVSLTCLVKGFYPSDIAVEWESNGQPENNYkTTPPVLDSDGSFFLYskLTVDKSRWQQGNVFSCSVMHEALHNHYTQK	K32 and K49 or K54	HC-K395 and HC-K412 or HC-K417	10921.03	30%	40.6	23%	40.7	31%	40.7	40%	40.5	23%	40.5
THTCPPCPAPELLGGPSVFLFPPkPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK	K24 or K26 and K52	HC-K249 or HC-K251 and HC-K277	9180.32			22%	42.0	#N/A	#N/A	26%	42.0	25%	42.1

Table S9. List of two payload-containing peptides identified with ProSight PD for EThcD replicates in secondary sample. For each peptide, the residue to which the payload was localized, the theoretical mass, as well as the sequence coverage and retention time for each replicate are listed. Some replicate entries are blank in the case that a peptide was not identified in all five technical replicates.

Annotated Sequence	Payload Localization (residue number on peptide sequence)	Payload Localization (residue number on protein sequence)	Theo. Mass [Da]	Replicate 1		Replicate 2		Replicate 3		Replicate 4		Replicate 5	
				SC	RT	SC	RT	SC	RT	SC	RT	SC	RT
AKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYkTTPPVLDSDGSFFLYskLTVDKSRWQQGNVFSVMHEALHNHYTQK	K54 and K71 or K76	HC-K395 and HC-K412 or HC-K417	13444.32	33%	38.4	40%	38.0	37%	38.2	38%	38.4	38%	38.3
EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGkGLEWVARIYPTNGYT RYADSVK	K30 and K43	HC-K30 and HC-K43	8977.34	23%	41.8	33%	41.7	17%	41.7	25%	41.9	28%	41.8
GFYPSDIAVEWESNGQPENNYkTTPPVLDSDGSFFLYSKLTVDkSRWQQGNVFSVMHEALHNHYTQK	K22 and K44	HC-K395 and HC-K417	9835.44	46%	40.5	63%	40.8	46%	40.5	49%	40.7	57%	40.5
NQVSLTCLVKGFYPSDIAVEWESNGQPENNYkTTPPVLDSDGSFFLYSKLTVDkSRWQQGNVFSVMHEALHNHYTQK	K32 and K49 or K54	HC-K395 and HC-K412 or HC-K417	10921.03	33%	40.5	39%	40.5	35%	40.4	32%	40.5	40%	40.4
THTCPPCPAPELLGGPSVFLFPPkPKDTLMISRTPEVTCVVVDVSHEDPEVkfFNWYVDGVEVHNAK	K24 or K26 and K52	HC-K249 or HC-K251 and HC-K277	9180.32	20%	42.0	43%	41.8	32%	41.8	39%	41.8	31%	41.8

Table S10. Number of identified payload-modified peptide spectral matches for two different lots of TDM-1 based on bottom-up analysis of tryptic digests.

Number of payload-modified peptide spectral matches		
	Lot #3535802	Lot #1153402
HCD only	21	46
HCD-triggered-EThcD	69*	48*
HCD-triggered-UVPD	75*	76*
No score cut-off is used		
* Includes peptide spectral matches (PSMs) from HCD as well.		

Table S11. Number of payload-modified lysines identified in heavy and light chains (HC and LC) of two different lots of TDM-1 based on bottom-up analysis of tryptic digests.

Number of modified Lysines (HC + LC)		
	Lot #3535802	Lot #1153402
HCD only	7 + 0	11 + 5
HCD-triggered-ETHcD	12 + 5 *	13 + 3 *
HCD-triggered-UVPD	14 + 4 *	15 + 5*
No score cut-off is used		
* Includes peptide spectral matches (PSMs) from HCD as well.		