

Characterization of Therapeutic Monoclonal Antibodies at the Subunit-Level using Middle-Down 193 nm Ultraviolet Photodissociation

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Supplemental Information: Supplement figures include a workflow diagram, representative chromatograms of mAb subunits, sequence alignment of mAbs, ESI-MS1 spectra of subunits, a bar graph summarizing the sequence coverage per subunit as a function of laser activation parameters, bar graphs comparing the number of positionally unique N-terminal and C-terminal fragment ions and total sequence coverage for adalimumab subunits, and tables summarizing the matched ions obtained by UVPD and two variations of ETD for all subunits.

Figure S1. Schematic representation of sample preparation and analysis workflow. IgG subunits are first produced from IdeS digestion and TCEP reduction. High resolution LC-MS¹ analysis using 120K resolution (at m/z 400) provides accurate mass measurements of subunits in addition to elution profiles and charge state distributions necessary for targeted MS/MS activation.

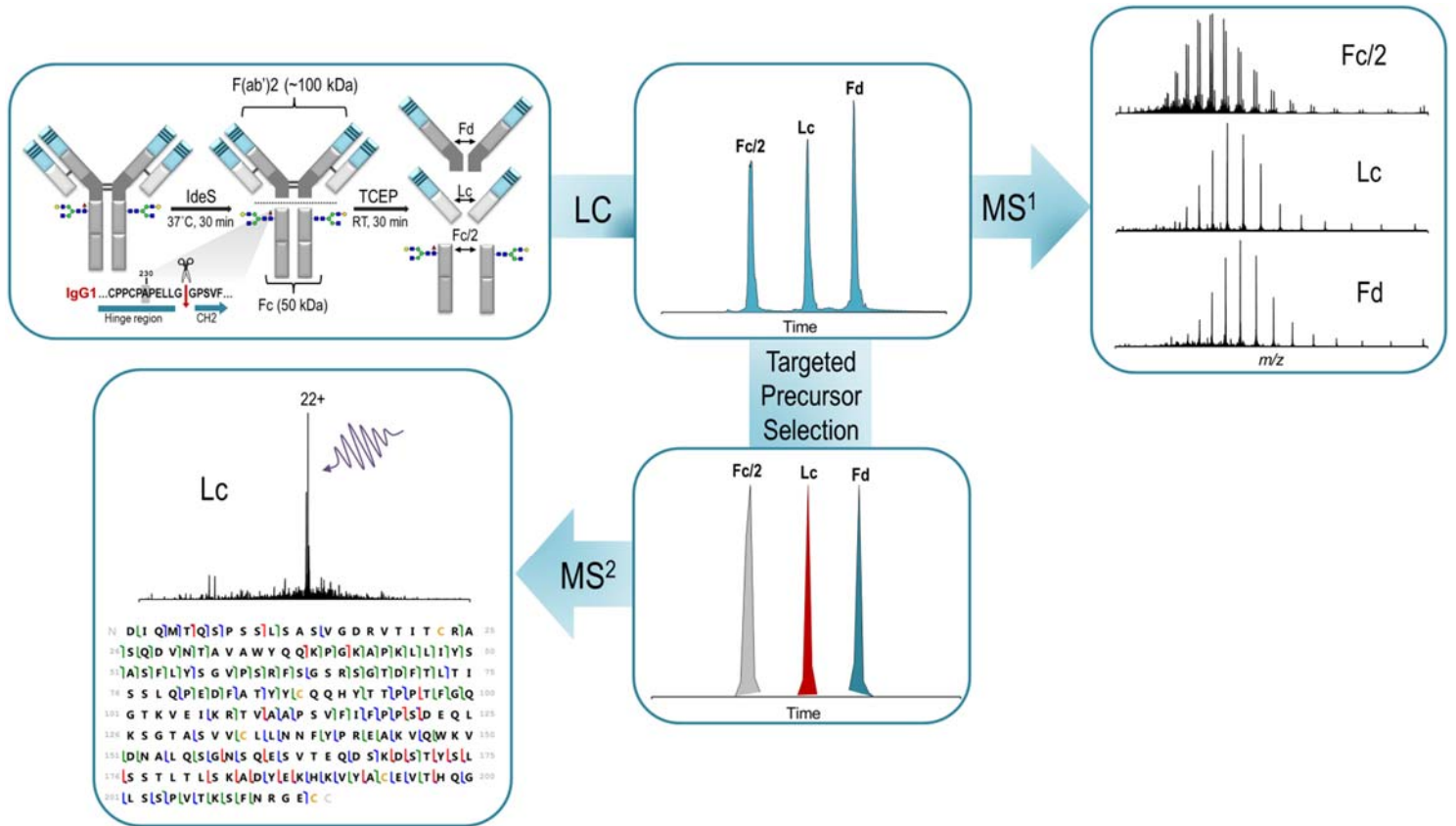


Figure S2. Stacked total ion chromatograms for triplicate LC-MS analyses of IdeS-derived (a) trastuzumab and (b) adalimumab subunits: Fc/2, Lc, and Fd, respectively, with baseline chromatographic resolution.

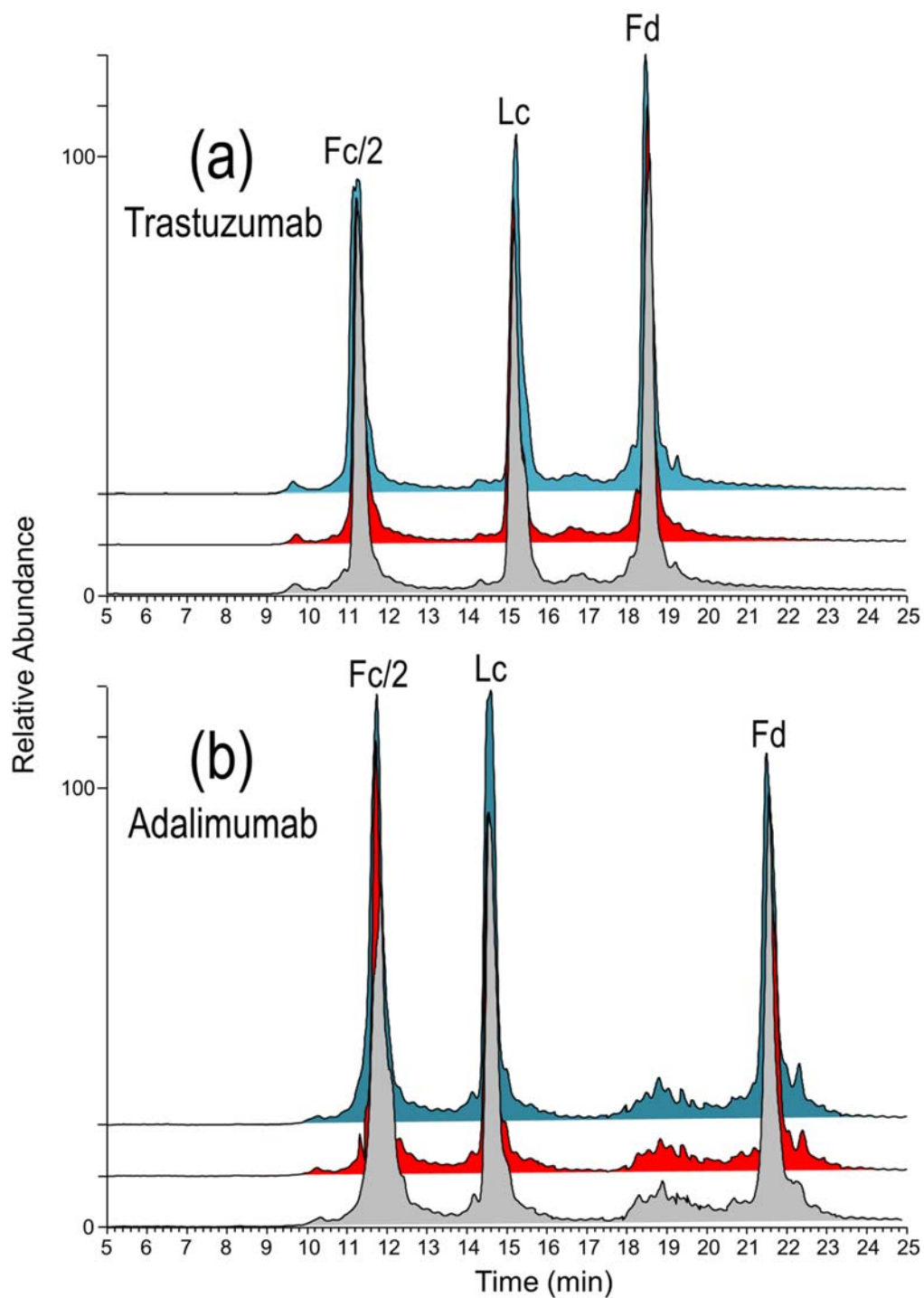


Figure S3. Sequence alignment of trastuzumab and adalimumab Fc/2, Lc, and Fd subunits. Hypervariable CDRs are shown in red.

Fc/2 subunit: 99% sequence identity

	10	20	30	40	50	60
Trastu	GPSVFLFPKPKD	TLMISRTPEVTCVVVDV	SHEDPEVKFNWYVDG	VEVHN	AKTKPREEQY	
Adalim	GPSVFLFPKPKD	TLMISRTPEVTCVVVDV	SHEDPEVKFNWYVDG	VEVHN	AKTKPREEQY	
	10	20	30	40	50	60
	70	80	90	100	110	120
Trastu	NSTYRVVSVLTVLHQD	WLNGKEYCKVSNKALP	APIEKTISKAKGQPREPQ	VYTLPPSRE		
Adalim	NSTYRVVSVLTVLHQD	WLNGKEYCKVSNKALP	APIEKTISKAKGQPREPQ	VYTLPPSRD		
	70	80	90	100	110	120
	130	140	150	160	170	180
Trastu	EMTKNQVSLTCLVKGF	YPSDIAVEWESNGQPENNY	KTTTPVLDSG	SFFLYSKLTVDKSR		
Adalim	ELTKNQVSLTCLVKGF	YPSDIAVEWESNGQPENNY	KTTTPVLDSG	SFFLYSKLTVDKSR		
	130	140	150	160	170	180
	190	200	210			
Trastu	WQQGNVFSCSVMHEAL	HNHYTQKSLSLSPG				
Adalim	WQQGNVFSCSVMHEAL	HNHYTQKSLSLSPG				
	190	200	210			

Lc subunit: 92% sequence identity

	10	20	30	40	50	60
Trastu	DIQMTQSPSSLSASVGD	RVTITCRASQDVNTAVAWYQ	QKPKGAPKLLIYSASFLY	SGVPS		
Adalim	DIQMTQSPSSLSASVGD	RVTITCRASQGIRN	LAWYQKPKGAPKLLIYA	AAS	TLQSGVPS	
	10	20	30	40	50	60
	70	80	90	100	110	120
Trastu	RFSGSRSGTDFTLTI	SSLPEDFATYYCQH	YTTPTFGQGT	KVEIKRTVAAPS	VFIFPP	
Adalim	RFSGSGSGTDFTLTI	SSLPEDVATYYCQ	RYNRAPYTFGQGT	KVEIKRTVAAPS	VFIFPP	
	70	80	90	100	110	120
	130	140	150	160	170	180
Trastu	SDEQLKSGTASVVC	LLNFPREAKVQWKVD	NALQSGNSQESVTE	QDSK	STYLS	SSLT
Adalim	SDEQLKSGTASVVC	LLNFPREAKVQWKVD	NALQSGNSQESVTE	QDSK	STYLS	SSLT
	130	140	150	160	170	180
	190	200	210			
Trastu	LSKADY	EKKVYACEVTHQGL	SSPVT	KSFNR	GEC	
Adalim	LSKADY	EKKVYACEVTHQGL	SSPVT	KSFNR	GEC	
	190	200	210			

Fd subunit: 86% sequence identity

	10	20	30	40	50	60
Trastu	EVQLVESGGGLVQP	GGSLRLSCAAS	GFNIKDTYIHWVRQ	APGKGLEWVAR	IYPTNGYTRY	
Adalim	EVQLVESGGGLVQP	GRSLRLSCAAS	GFTFDDYAMHWVRQ	APGKGLEWVS	AITWNSGHIDY	
	10	20	30	40	50	60
	70	80	90	100	110	
Trastu	ADSVKGRETI	SADTSKNTAYLQ	MNSLRAEDTAV	YCSRWGG-DG	FYAMDYWGQ	GLTVTS
Adalim	ADSVEGRETI	SISRDNAKNSL	YLQMSLRAEDT	AVYYCAKVS	YLSLASSLD	YWGQGLTVTS
	70	80	90	100	110	120
	120	130	140	150	160	170
Trastu	SASTKGPSVF	FLAPSSKSTSG	GTAAALGCLV	KDYFPEPVT	SWNSGALTS	GVHFTFAVLQS
Adalim	SASTKGPSVF	FLAPSSKSTSG	GTAAALGCLV	KDYFPEPVT	SWNSGALTS	GVHFTFAVLQS
	130	140	150	160	170	180
	180	190	200	210	220	230
Trastu	SGLYSLSSV	VTVPSSSLGT	QTYICNVNHK	PSNTKVDKK	VEPKSCDKT	HTCPCPAPELLG
Adalim	SGLYSLSSV	VTVPSSSLGT	QTYICNVNHK	PSNTKVDKK	VEPKSCDKT	HTCPCPAPELLG
	190	200	210	220	230	240

Figure S4. ESI mass spectra for the Fc/2, Lc, and Fd subunits of trastuzumab (a-c) and adalimumab (d-f), respectively, collected at 120K resolution (at m/z 400). The insets for the Fc/2 subdomains demonstrate the glycoform heterogeneity in each IgG based on accurate mass measurement. Trastuzumab exhibited the G0, G0F, G1F, and G2F glycoforms (a), whereas adalimumab exhibited the G0F and G1F variants only. The insets for all other subunits demonstrate the isotope distribution for the most abundant charge state.

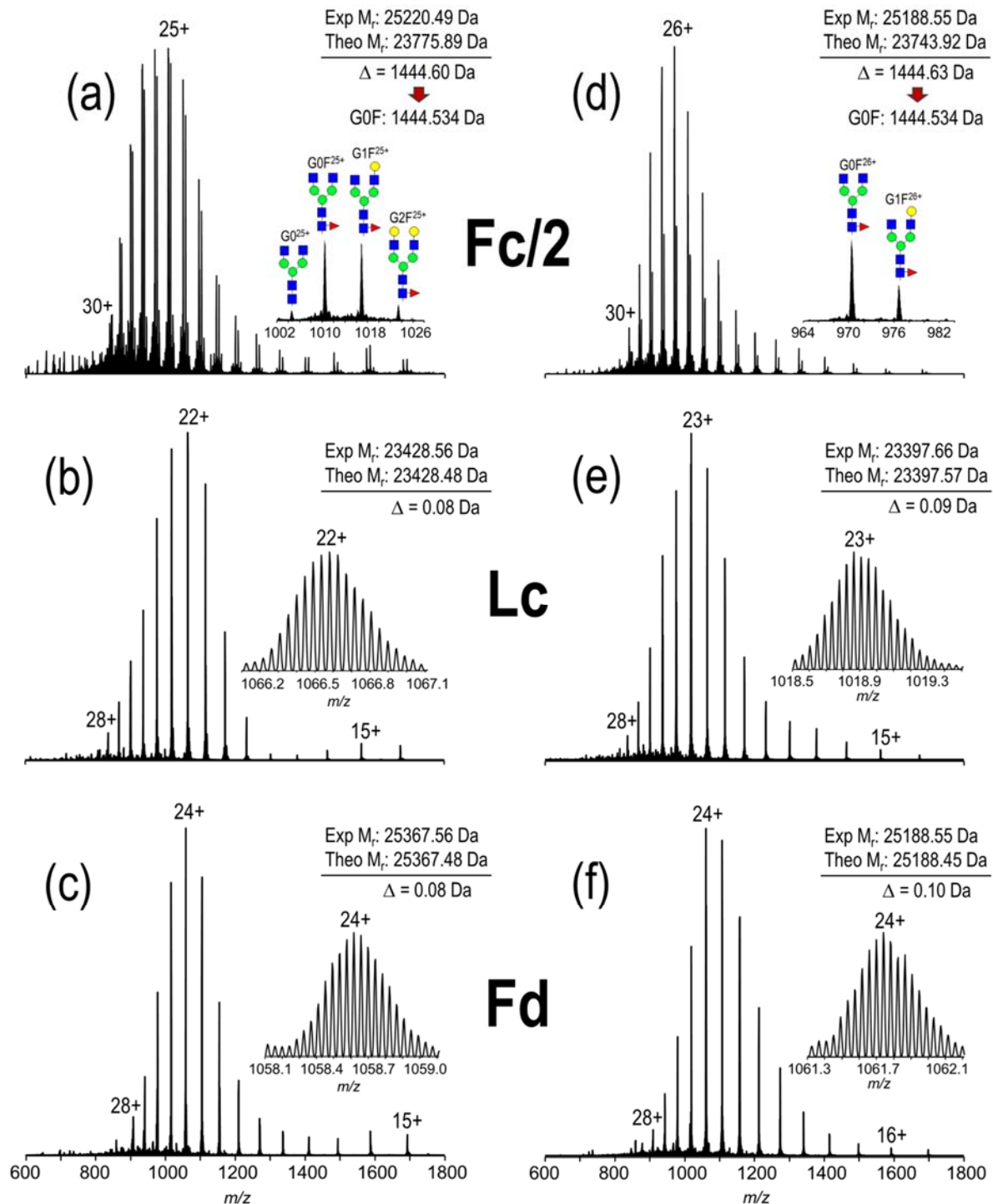


Figure S5. Sequence coverage observed as a function of laser parameter selection used for targeted UVPD of the most abundant precursor of the Fc/2 (25+), Lc (22+) and Fd (24+) subunits of trastuzumab.

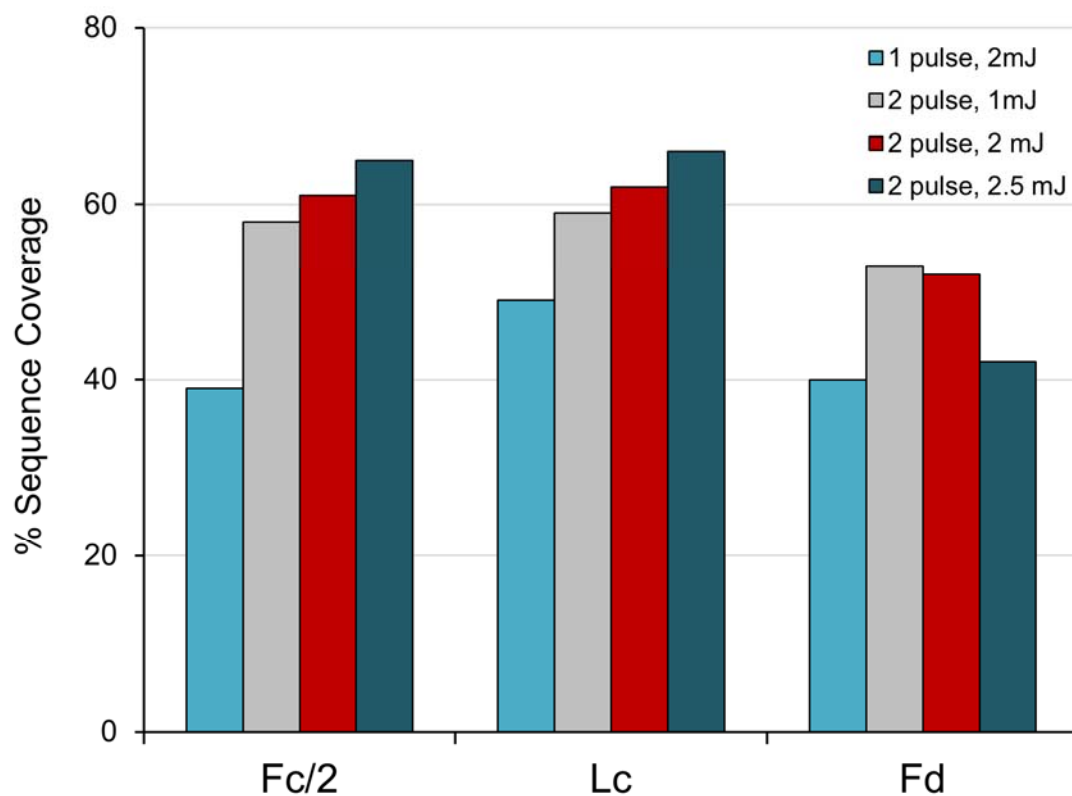


Figure S6. Evaluation of unique fragment ions and sequence coverage obtained from a targeted LC-MS/MS analysis of adalimumab subunits based on UVPD (20 m/z isolation, most abundant charge state), ETD (5 ms reaction time) using single precursor isolation (20 m/z isolation, the most abundant charge state), and ETD (5 ms reaction time) using multiple precursor isolation (150 m/z isolation, high charge states). The most abundant charge states were as follows: +26 for Fc/2, +23 for Lc, and +24 for Fd. The isolation range used for multiple precursor isolation included: +26 to +30 for Fc/2, +25 to +27 for Lc, and +25 to +28 for Fd. The fourth bar (shaded purple) in the second bar graph shows the net sequence coverage for combined product ion information from UVPD and broadband ETD.

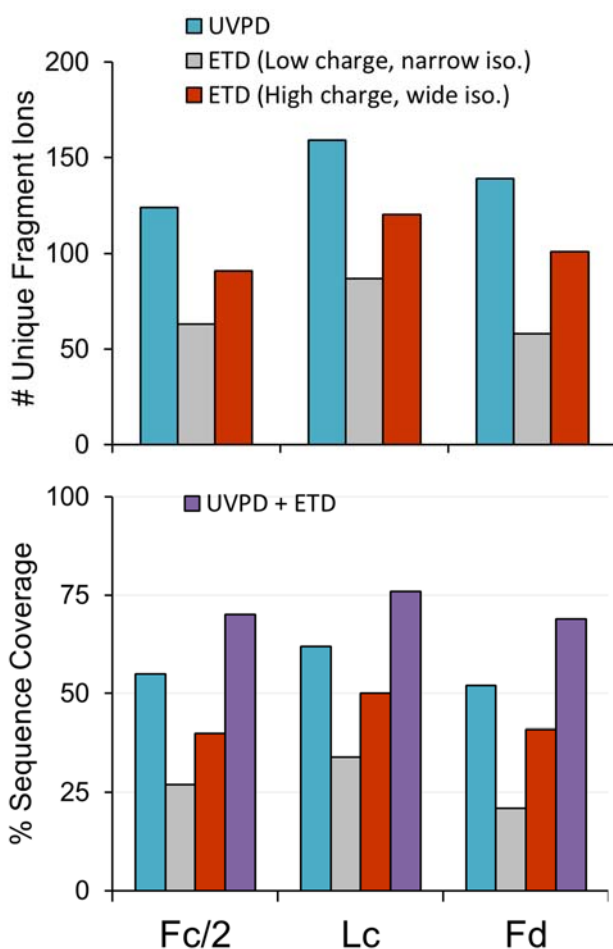


Figure S7. Fragment ion maps for the Fc/2 subunit of trastuzumab generated by a single LC-MS/MS experiment based on UVPD (top left) and broadband ETD (top right) and a composite map (bottom) generated by combining the fragment ion information from both experiments.

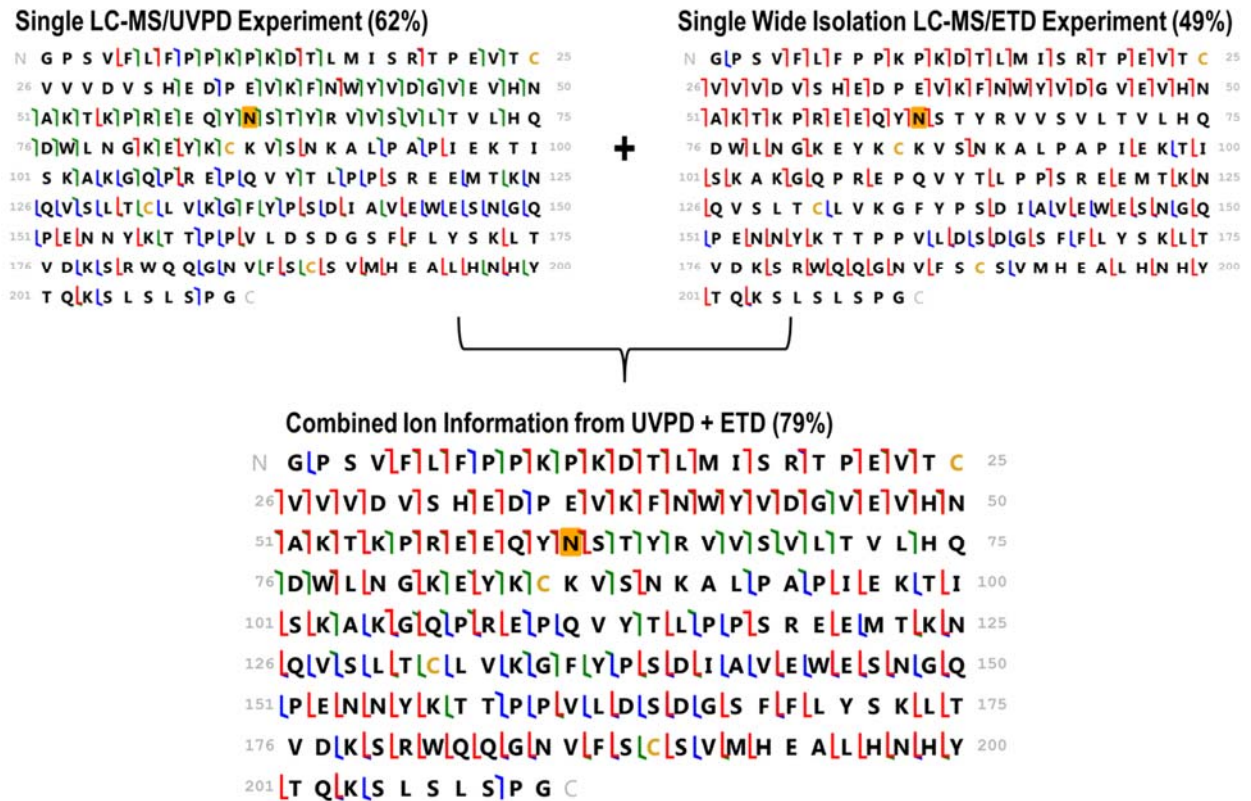


Figure S8. Zoomed-in view of spectral region spanning from 880-900 m/z for Fd subunit of trastuzumab following isolation of the most abundant precursor ion (24+) obtained using (a) UVPD (2 pulses, 2 mJ) and (b) ETD (5 ms reaction time), respectively, and (c) ETD (5 ms reaction time) with wide isolation (150 m/z) centered at the 27+ charge state.

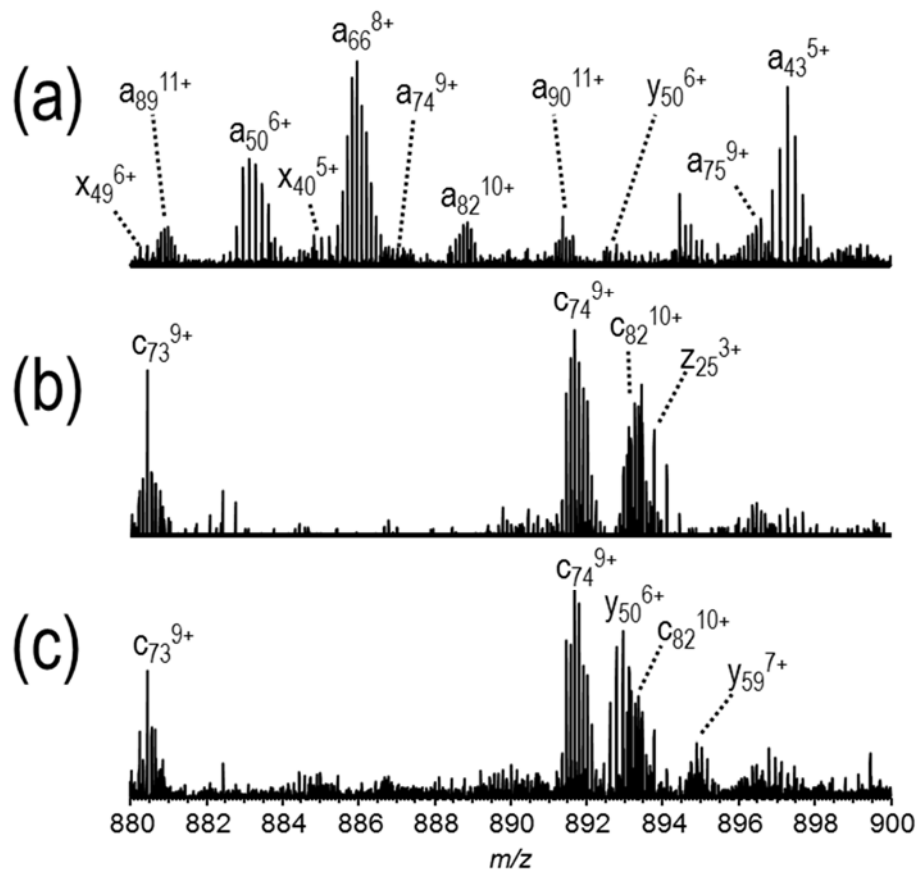


Table S1. List of confidently identified fragment ions (10 ppm mass tolerance, $S/N \geq 3$) for the Fc/2 (Sheet 1), Lc (Sheet 2), and Fd (Sheet 3) subunits of trastuzumab using: UVPD (20 m/z isolation, most abundant charge state), ETD (5 ms reaction time) using single precursor isolation (20 m/z isolation, the most abundant charge state), and ETD (5 ms reaction time) using multiple precursor isolation (150 Th isolation, high charge states). The most abundant charge states were as follows: +25 for Fc/2, +22 for Lc, and +24 for Fd. The isolation range used for multiple precursor isolation included: +25 to +31 for Fc/2, +24 to +28 for Lc, and +25 to +29 for Fd.

Table S2. List of confidently identified fragment ions (10 ppm mass tolerance, $S/N \geq 3$) for the Fc/2 (Sheet 1), Lc (Sheet 2), and Fd (Sheet 3) subunits of adalimumab using: UVPD (20 m/z isolation, most abundant charge state), ETD (5 ms reaction time) using single precursor isolation (20 m/z isolation, the most abundant charge state), and ETD (5 ms reaction time) using multiple precursor isolation (150 Th isolation, high charge states). most abundant charge states were as follows: +26 for Fc/2, +23 for Lc, and +24 for Fd. The isolation range used for multiple precursor isolation included: +26 to +30 for Fc/2, +25 to +27 for Lc, and +25 to +28 for Fd.