

## Supplementary Information

Selective detection of complementarity-determining regions of monoclonal antibody by limiting protease access to the substrate: nano-surface and molecular-orientation limited proteolysis

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## Experimental details

### Reagents

Glycidyl methacrylate (GMA) coated nano-ferrite particle with N-hydroxysuccinimide (NHS) activated FG beads was purchased from Tamagawa Seiki (Nagano, Japan). Protein G Ultralink resin was from Thermo Fisher Scientific (Rockford, IL, USA). Trastuzumab and Bevacizumab were purchased from Chugai Pharmaceutical (Tokyo, Japan) and F. Hoffmann-La Roche (Basel, Switzerland), respectively. Mass spectrometry grade Trypsin Gold and recombinant Lys-C (rLys-C) were from Promega (Fitchburg, WI, USA). 2-Morpholinoethanesulfonic acid (MES), 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) and *n*-octyl- $\beta$ -D-thioglucopyranoside (OTG) were from Dojindo Laboratories (Kumamoto, Japan). Recrystallized high-purity MALDI matrix 2,5-dihydroxybenzoic acid (DHB) was from Shimadzu GLC (Tokyo, Japan). Other reagents, buffers, and solvents were from Sigma-Aldrich (St. Louis, MO, USA) and Wako Pure Chemical Industries (Osaka, Japan).

### Preparation of FG protease beads

One hundred  $\mu\text{g}$  of Trypsin Gold, rLys-C, and mixed protease were each dissolved in 200  $\mu\text{l}$  of 25 mM HEPES-NaOH (pH 7.0). FG beads were washed with cold methanol under suspension in ultrasonic water bath and centrifuged at 20,000 g for 5 min. Protease solution was added to the FG beads, and the protease was immobilized to FG under suspension at 4 °C for 3 h with gentle vortexing. The quantity of the immobilized protease on FG beads was 50  $\mu\text{g}/\text{mg}$ . Protease solution was removed with centrifugation, and excess NHS group was blocked with 1 M ethanolamine (pH 8.0) at 4 °C for 60 min. FG protease beads were washed twice with 50 mM Tris-HCl containing 1 mM calcium chloride (pH 8.0), and stored at -80 °C until use.

### Limited proteolysis of antibody

One hundred  $\mu\text{g}$  of Trastuzumab or Bevacizumab solution (20 mg/ml) was dissolved in 200  $\mu\text{l}$  of 25 mM MES-NaOH (pH 5.5). Ten  $\mu\text{l}$  of Protein G Ultralink resin slurry was added to the antibody solution, and the antibody Fc region was immobilized to the resin

at 25 °C for 60 min with gentle vortexing. Antibody-immobilized resin was washed with 25 mM Tris-HCl containing 1 mM calcium chloride (pH 8.0), and limited proteolysis was performed at 37 °C for 6 h with antibody-to-immobilized protease ratio of 9:1 (w/w). The protease trypsin/Lys-C ratio was set to 10/0, 9/1, and 0/10. Generated peptides were collected by centrifugation.

### **MALDI mass spectrometry**

Peptides generated by limited proteolysis were purified using ZipTip  $\mu$ C18 for matrix-assisted laser desorption/ionization-quadrupole ion trap time-of-flight MS analysis (MALDI-QIT-TOF MS) (AXIMA Resonance, Shimadzu, Kyoto, Japan). For MALDI MS condition, the digested solution was acidified with trifluoroacetic acid to a final concentration of 0.5%. ZipTip  $\mu$ C18 elution was directly applied onto a MALDI stainless target. After drying the sample, 10 mg/ml of DHB was overlaid to the sample spot. The instrument was externally calibrated by angiotensin II ( $m/z$  1046.54) and ACTH fragment ( $m/z$  2465.20). MALDI MS spectra were obtained in mid-mass and positive ion mode, with 337 nm nitrogen laser and an accelerating voltage of 20 kV. Peptides were assigned by peptide mass fingerprinting against the non-redundant human protein database of National Center for Biotechnology Information (NCBI) and in-house sequence database of monoclonal antibody in Mascot server (Matrix Science, London, UK) using monoisotopic peak lists from the obtained MALDI MS data. Assigned peptides in Trastuzumab and Bevacizumab CDR region were confirmed continuously by MALDI MS/MS analysis.

### **Selection of peptides for Trastuzumab quantitation by multiple reaction monitoring**

For LC/MS condition, digested solution was acidified with formic acid to a final concentration of 0.5%. Peptides were analyzed by LC-electrospray ionization-MS (LC-ESI-MS) with triple quadrupole (Nexera X2 ultra high performance liquid chromatograph and LCMS-8080, Shimadzu). LC conditions were as follows: solvent A, 0.1% formic acid and 1% acetonitrile; solvent B, 0.1% formic acid and acetonitrile; 2.0 x 50 mm Shim-pack XR ODS-II column (Shimadzu); column temperature, 40 °C; flow rate, 0.4 ml/min; gradient program, 0-2 min: %B=0, 2-10 min: %B=0-40 gradient, 10-11

min: %B=40-98 gradient, 11-13 min: %B=98, 13-13.5 min: %B=98-0 gradient, 13.5-15 min: %B=0. MS spectra were obtained with the ESI probe temperature and desolvation line at 350 °C and 300 °C, respectively. Nebulizer, heating, and curtain nitrogen gas flows were set to 2, 12, and 3 liter/min, respectively. The retention time and fragment ion series from each peptide were assigned by product ion scan with optimized electrode voltage of collision cell Q2 (Supplementary Figure 2). The dwell time was set to 100 ms for each transition. For MRM channel conditions, 2 fragment ions of b- or y-series were selected for each peptide to be quantified and confirmed. The electrode voltage of collision cell lens 4 (CCL4) was optimized to the voltage where the signal intensity of the peptide sequence-specific fragment ion is maximized (Supplementary Table 2).

#### **Trastuzumab quantitation in plasma**

The immunoglobulin fraction contained within 10 µl of Trastuzumab-spiked human plasma (50 mg/ml plasma protein concentration) was collected with 30 µl of Protein G Ultralink resin slurry. Plasma was diluted 10-fold by phosphate-buffered saline (pH 7.4) containing 0.1% OTG. The final concentrations of Trastuzumab in plasma were 500, 100, 20, 5, 2.5, and 1 µg/ml. Protein G resin was washed 3-times by PBS containing OTG and substituted with 25 mM Tris-HCl containing 1 mM calcium chloride (pH 8.0). nSMOL proteolysis was carried out using 20 µg protease with 9:1 trypsin/Lys-C ratio. MRM analysis was performed as described above. MRM quantified data was obtained from triplicate analyses of 6-time proteolysis batches each. LOD was defined at the concentration at RSD above 0.5 or at which the obtained data points were below 6.

## Supplementary Figure legend

Supplementary Fig. 1. Effect of antibody fragmentation by trypsin/Lys-C double digestion in nSMOL proteolysis.

Trastuzumab and Bevacizumab on Protein G beads were digested by nSMOL methods, and the remaining antibodies on the beads were separated by non-reducing SDS-PAGE. The molecular weight marker lane is shown on the far left. Left box, from the left lane: 0.5 mg/ml Trastuzumab control, Trastuzumab remaining on Protein G beads with trypsin/Lys-C ratio of 10/0, 9/1, and 0/10. The right box shows the corresponding results for Bevacizumab. Solid arrow indicates the full length antibody ("Full"), and dashed arrow indicates the Fc region ("Fc"). Table below lists the contents of Trastuzumab and Bevacizumab fragments measured by densitometric estimation.

Supplementary Fig. 2. The effect of Fab-selective proteolysis by nSMOL method against a solution digestion.

MALDI MS spectra of digested peptides from the therapeutic antibody on immobilized Protein G resin were indicated. a) Trastuzumab peptides digested by nSMOL method, b) Trastuzumab peptides by the normal solution protease, c) Bevacizumab by nSMOL, and d) Bevacizumab by solution protease. All procedure was used the equal antibody and protease quantity, and changed the ratio of trypsin/ LysC content as 10/0, 9/1, and 0/10. The red asterisk shows the ion signal of peptides from Fab region, and black arrows are from Fc region.

Supplementary Fig. 3. Product ion spectra of selected Trastuzumab peptides for quantitation by MRM.

Product ion spectra of a) DTYIHWVR ( $m/z$  363.8), b) ASQDVNTAVAWYQQKPGK ( $m/z$  664.3), and c) IYPTNGYTR ( $m/z$  542.8), with assigned ion series using LCMS-8080. Precursor ion is indicated at the top right.

Supplementary Fig. 4. Calibration curves of Trastuzumab concentration in plasma from MRM quantitation using the fragment ion of Trastuzumab CDR peptide.

Calibration curves by MRM quantitation are shown for the following peptides a)

DTYIHWVR: fragment ion  $m/z$  437.2 (precursor ion  $m/z$  363.8) as  $y_6^{2+}$  ion, b)  
ASQDVNTAVAWYQQKPGK: fragment ion  $m/z$  852.9 (precursor ion  $m/z$  664.3) as  $y_{15}^{2+}$ ,  
c) IYPTNGYTR: fragment ion  $m/z$  808.4 (precursor ion  $m/z$  542.8) as  $y_7$  ion, and ion  
with good linearity. The error bars indicate standard deviation from 12 technical  
replicates.

Supplementary Table 1a. Detected peptides from Trastuzumab are listed by nSMOL proteolysis with sequence position, observed m/z, calculated m/z, mass shift, amino acid sequence, domain from each chain, and region.

Trypsin/LysC ratio=9/1						
Position	Observed m/z	Calculated m/z	Delta [Da]	Sequence	Domain	Region
1-19	1881.95	1881.00	-0.054	EVQLVESGGGLVQPGGSLR	VH	Fab
31-38	1089.51	1088.54	-0.039	DTYIHWVR	VH	CDR1
44-50	830.41	829.44	-0.038	GLEWVAR	VH	CDR2
51-59	1084.51	1083.53	-0.026	IYPTNGYTR	VH	CDR2
66-76	1182.57	1181.60	-0.037	GRFTISADTSK	VH	Fab
68-76	969.44	968.48	-0.045	FTISADTSK	VH	Fab
77-87	1310.61	1309.65	-0.042	NTAYLQMNSLR	VH	Fab
99-124	2784.17	2783.25	-0.093	WGGDGFYAMDYWGQGLVTVSSASTK	VH	CDR3
125-136	1186.61	1185.64	-0.040	GPSVFPLAPSSK	CH1	Fab
253-259	835.39	834.43	-0.040	DTLMISR	CH2	Fc
279-292	1677.75	1676.80	-0.050	FNWYVDGVEVHNAK	CH2	Fc
306-321	1807.96	1807.00	-0.050	VVSVLTVLHQDWLNGK	CH2	Fc
375-396	2544.07	2543.12	-0.063	GFYPSDIAVEWESNGQPENNYK	CH3	Fc
397-413	1873.87	1872.92	-0.053	TTPPVLDSDGSFFLYSK	CH3	Fc
1-18	1878.76	1877.88	-0.120	DIQMTQSPSSLSASVGDR	VL	Fab
25-42	1990.94	1989.99	-0.054	ASQDVNTAWWYQQKPGK	VL	CDR1
46-61	1772.90	1771.95	-0.053	LLIYSASFLYSGVPSR	VL	CDR2
108-126	2102.07	2101.12	-0.061	RTVAAPSVFIFPPSDEQLK	VL	Fab
109-126	1945.97	1945.02	-0.054	TVAAPSVFIFPPSDEQLK	CL	Fab
150-169	2135.90	2134.96	-0.068	VDNALQSGNSQESVTEQDSK	CL	Fab
170-183	1502.71	1501.75	-0.046	DSTYLSSTLTLSK	CL	Fab
Trypsin/LysC ratio=0/10						
Position	Observed m/z	Calculated m/z	Delta [Da]	Sequence	Domain	Region
31-43	1570.71	1569.81	-0.107	DTYIHWVRQAPGK	VH	CDR1
44-65	2559.13	2558.29	-0.168	GLEWVARIYPTNGYTRYADSVK	VH	CDR2
66-76	1182.54	1181.60	-0.065	GRFTISADTSK	VH	Fab
125-136	1186.57	1185.64	-0.081	GPSVFPLAPSSK	CH1	Fab
253-278	2898.23	2897.42	-0.192	DTLMISRTPEVTCVVVDVSHEDPEVK	CH2	Fc
279-292	1677.69	1676.80	-0.108	FNWYVDGVEVHNAK	CH2	Fc
331-338	838.43	837.50	-0.069	ALPAPIEK	CH2	Fc
375-396	2543.99	2543.12	-0.139	GFYPSDIAVEWESNGQPENNYK	CH3	Fc
397-413	1873.80	1872.92	-0.120	TTPPVLDSDGSFFLYSK	CH3	Fc
108-126	2101.99	2101.12	-0.140	RTVAAPSVFIFPPSDEQLK	CL	Fab
150-169	2135.83	2134.96	-0.137	VDNALQSGNSQESVTEQDSK	CL	Fab
170-183	1502.66	1501.75	-0.103	DSTYLSSTLTLSK	CL	Fab

Supplementary Table 1b. Detected peptides from Bevacizumab are listed as described in Table 1a.

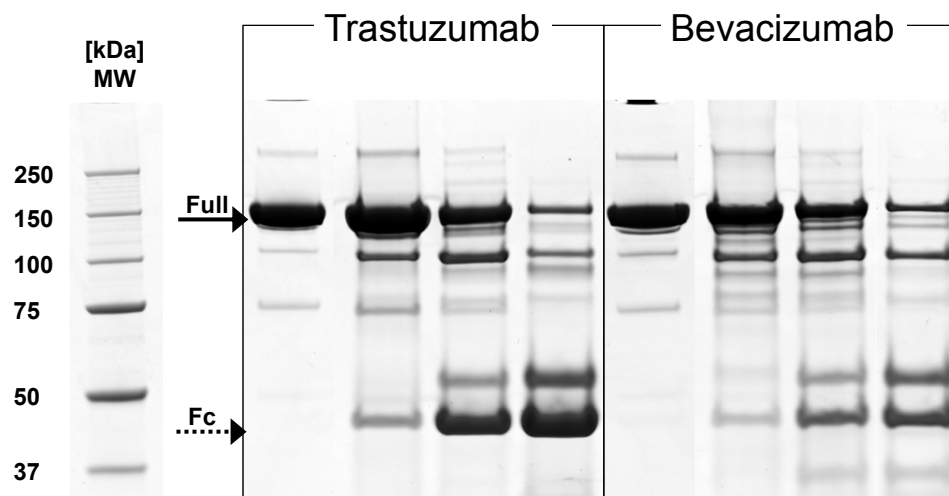
Trypsin/LysC ratio=9/1						
Position	Observed m/z	Calculated m/z	Delta [Da]	Sequence	Domain	Region
1-19	1881.87	1881.00	-0.136	EVQLVESGGGLVQPGGSLR	VH	Fab
44-65	2518.00	2517.19	-0.190	GLEWVGWINTYTGEPTYAADFK	VH	CDR2
44-66	2674.09	2673.29	-0.201	GLEWVGWINTYTGEPTYAADFKR	VH	CDR2
67-76	1201.53	1200.61	-0.087	RFTFSLDTSK	VH	Fab
68-76	1045.44	1044.51	-0.076	FTFSLDTSK	VH	Fab
77-87	1283.55	1282.63	-0.092	STAYLQMNSLR	VH	Fab
128-139	1186.56	1185.64	-0.088	GPSVFPLAPSSK	CH1	Fab
255-261	835.37	834.43	-0.066	DTLMISR	CH2	Fc
281-294	1677.68	1676.80	-0.121	FNWYVDGVEVHNAK	CH2	Fc
308-323	1807.88	1807.00	-0.131	VVSVLTVLHQDWLNGK	CH2	Fc
1-18	1878.76	1877.88	-0.120	DIQMTQSPSSLSASVGDR	VL	Fab
46-61	1762.81	1761.94	-0.128	VLIYFTSSLSHSGVPSR	CL	CDR2
108-126	2101.96	2101.12	-0.159	RTVAAPSVFIFPPSDEQLK	CL	Fab
109-126	1945.88	1945.02	-0.144	TVAAPSVFIFPPSDEQLK	CL	Fab
170-183	1502.65	1501.75	-0.109	DSTYLSSTLTLSK	CL	Fab
Trypsin/LysC ratio=0/10						
Position	Observed m/z	Calculated m/z	Delta [Da]	Sequence	Domain	Region
44-65	2518.04	2517.19	-0.156	GLEWVGWINTYTGEPTYAADFK	VH	CDR2
66-76	1357.64	1356.72	-0.080	RRFTFSLDTSK	VH	Fab
128-139	1186.57	1185.64	-0.074	GPSVFPLAPSSK	CH1	Fab
255-280	2898.23	2897.42	-0.196	DTLMISRTPEVTCVVVDVSHEDPEVK	CH2	Fab
281-294	1677.70	1676.80	-0.102	FNWYVDGVEVHNAK	CH2	Fc
333-340	838.45	837.50	-0.057	ALPAPIEK	CH2	Fc
347-366	2343.02	2342.17	-0.155	GQPREPQVYTLPPSREEMTK	CH3	Fc
399-415	1873.81	1872.92	-0.117	TTPPVLDSDGSFFLYSK	CH3	Fc
108-126	2102.00	2101.12	-0.130	RTVAAPSVFIFPPSDEQLK	CL	Fab
170-183	1502.67	1501.75	-0.093	DSTYLSSTLTLSK	CL	Fab



Supplementary Table 2. Selected MRM peptides and optimal electrode conditions for Trastuzumab quantitation. The parameters are defined as follows: Selected peptide; peptide sequence for Trastuzumab quantitation, Region in Trastuzumab; region of each peptide, Transition mass filter; fragment ion  $m/z$  for quantitation from the parent ion  $m/z$ , Collision cells [V]; voltage condition of the collision cell Q2, CCL4; electrode voltage of collision cell lens 4 after quadrupole 2 collision cell, Role; purpose of each ion  $m/z$ .

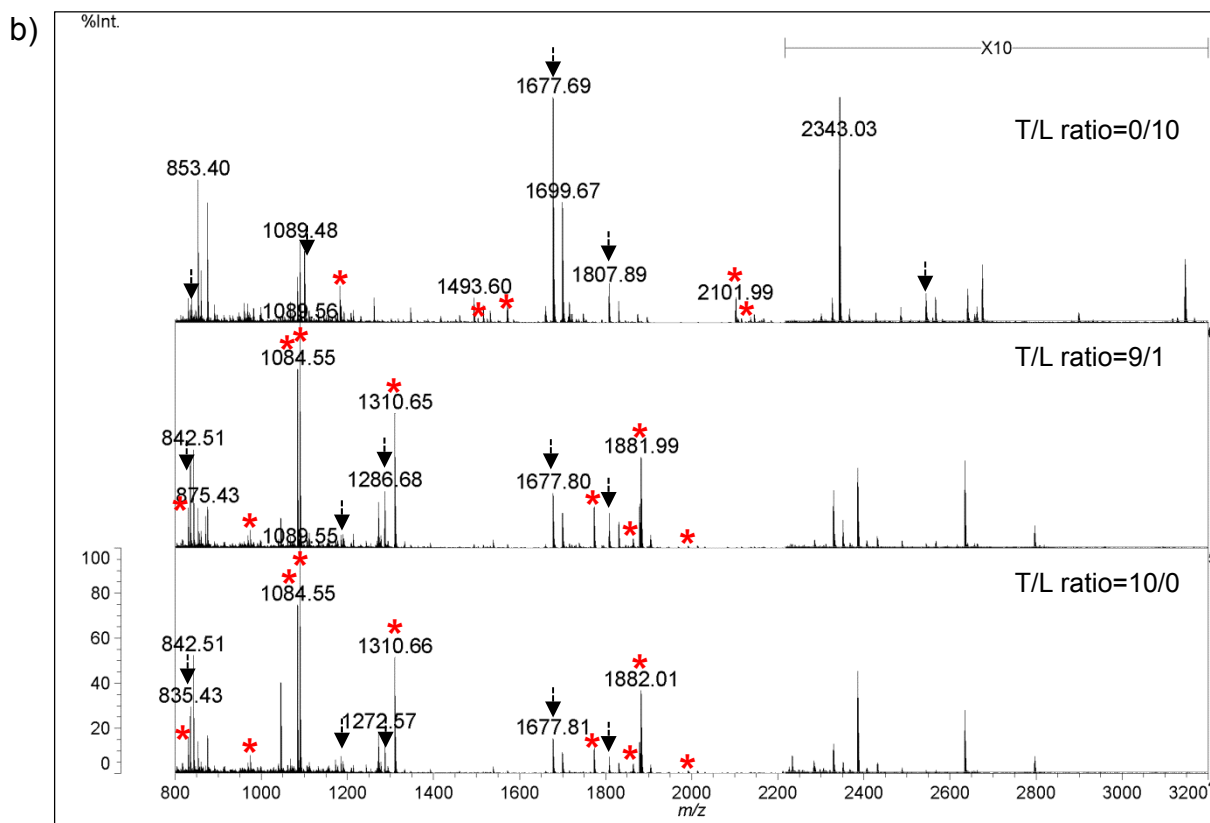
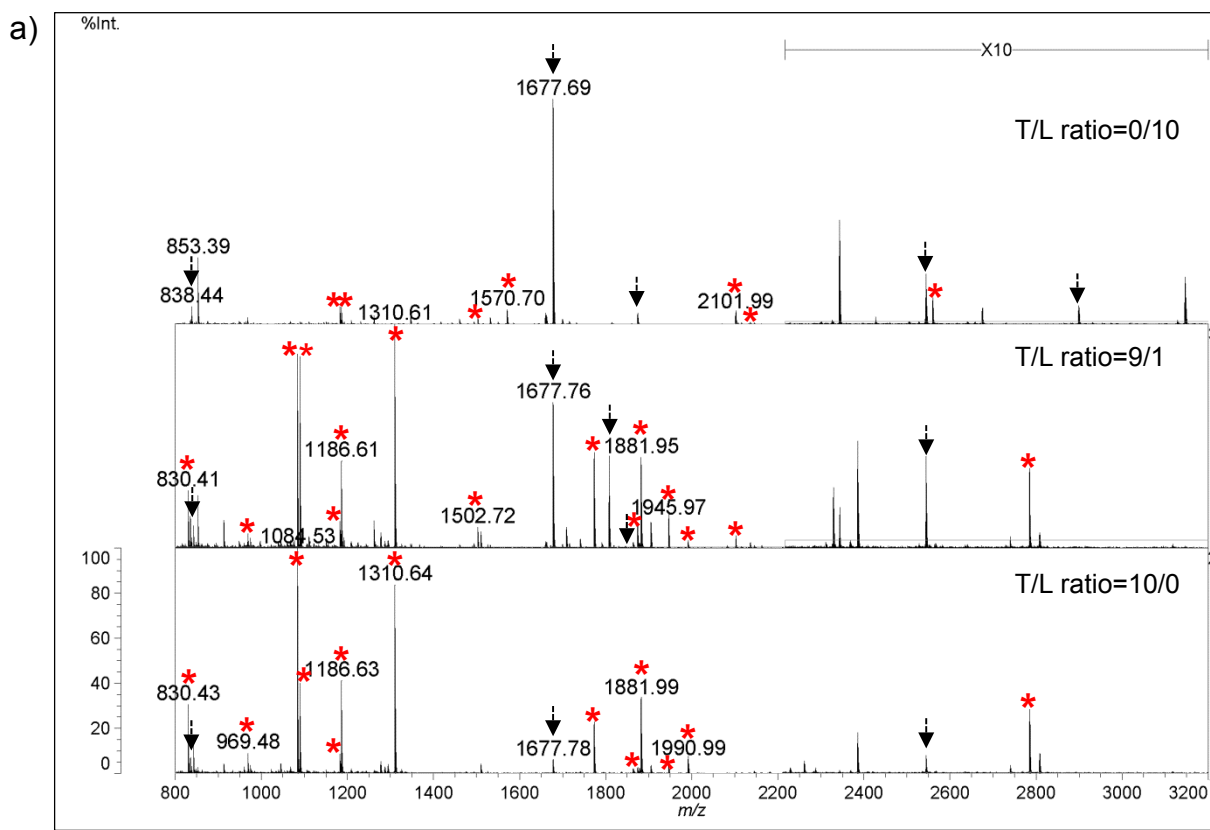
Selected peptide	Region in Trastuzumab	Optimal MRM condition			Role
		Transition mass filter [ $m/z$ ]	Collision cell [V]	CCL4 [V]	
DTYIHWVR	H-chain of	363.8→437.2 (y6 <sup>2+</sup> )	-8	-11	Quantitation
	CDR1	363.8→299.2 (y4 <sup>2+</sup> )	-12	-8	Confirmation
ASQDVNTAVAWYQQKPGK	L-chain of	664.3→852.9 (y15 <sup>2+</sup> )	-14	-26	Quantitation
	CDR1	664.3→917.0 (y16 <sup>2+</sup> )	-13	-28	Confirmation
IYPTNGYTR	H-chain of	542.8→808.4 (y7 <sup>+</sup> )	-13	-25	Quantitation
	CDR2	542.8→277.2 (b2 <sup>+</sup> )	-12	-8	Confirmation

# Supplementary Fig. 1

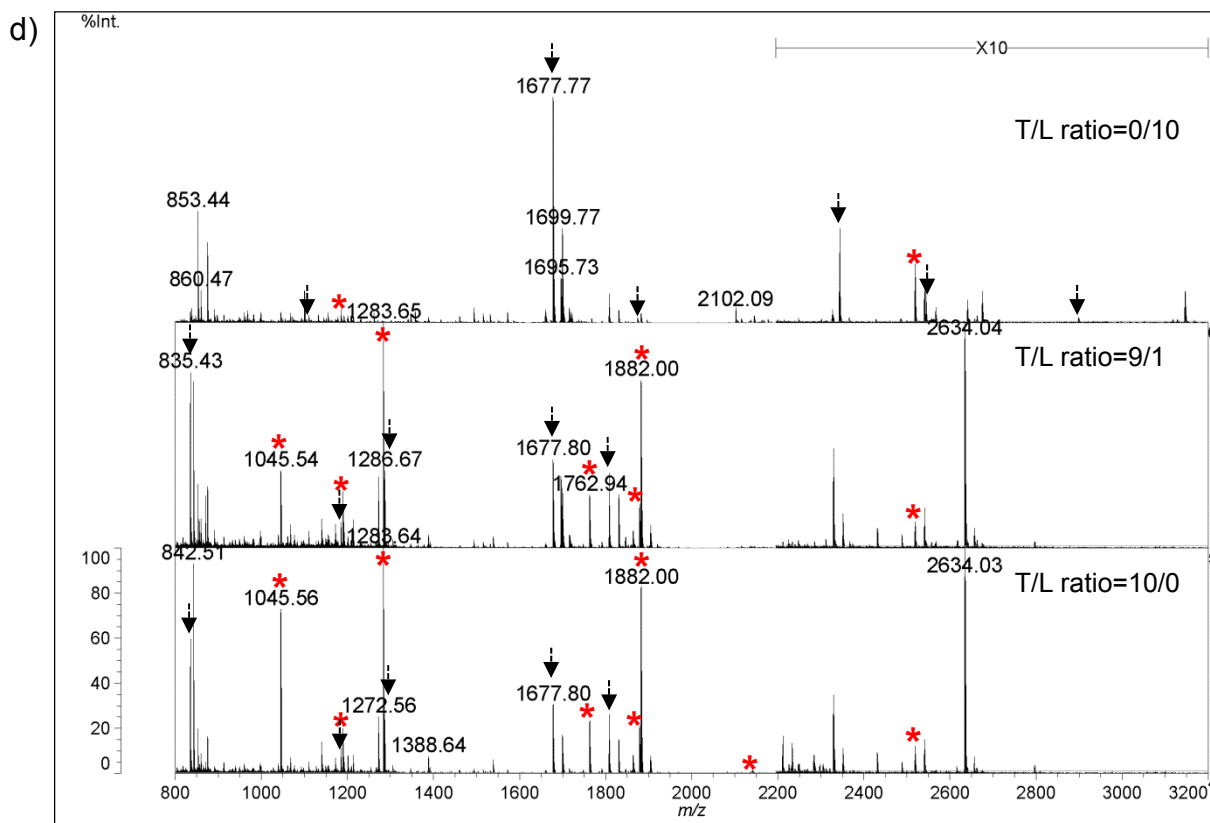
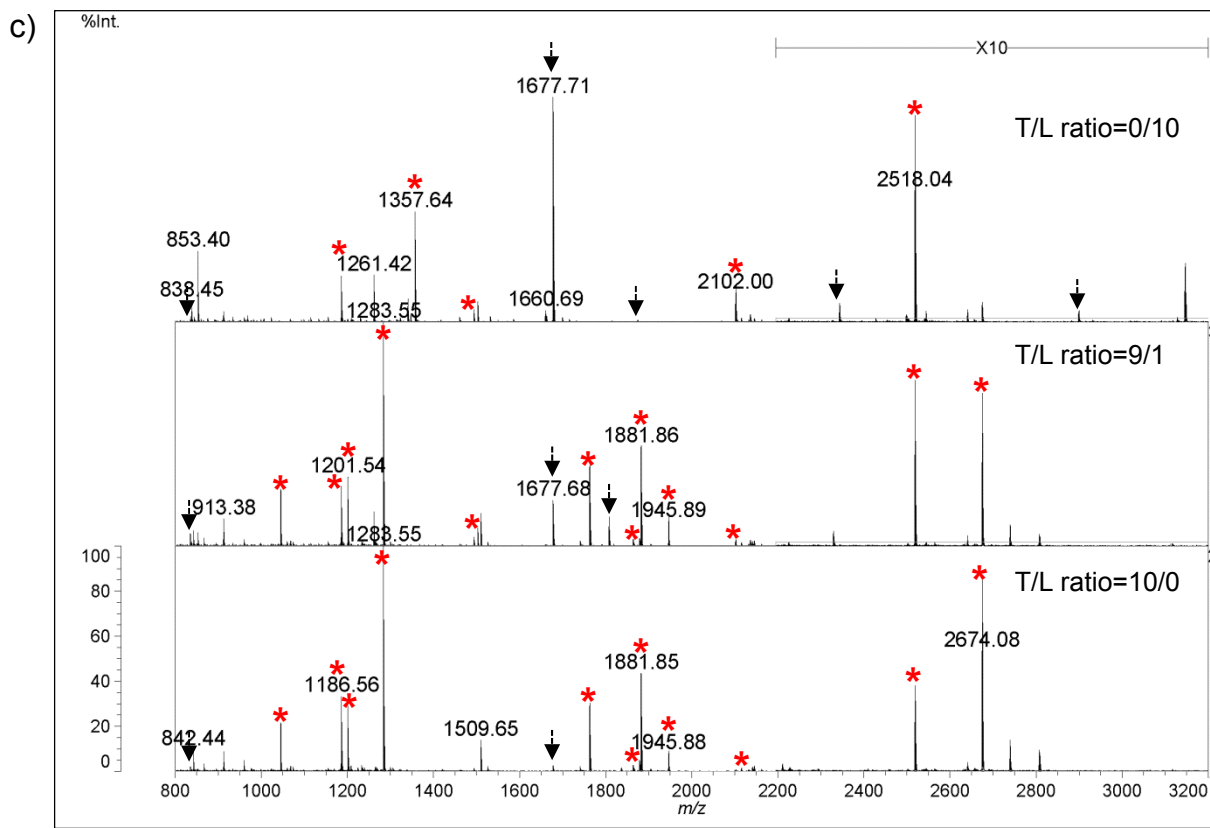


Trypsin/Lys-C ratio	10/0	9/1	0/10	10/0	9/1	0/10
Density (Full length + Fc)	253	229	179	200	179	117
Content % (Full length)	89	54	21	90	69	40
Content % (Fc)	11	46	79	10	31	60

## Supplementary Fig. 2

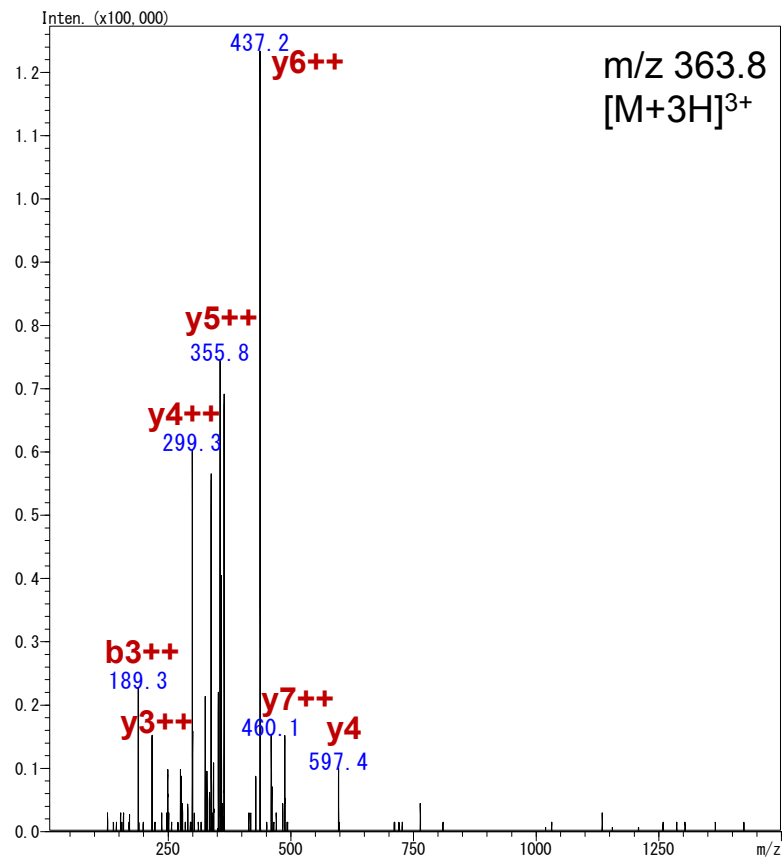


## Supplementary Fig. 2

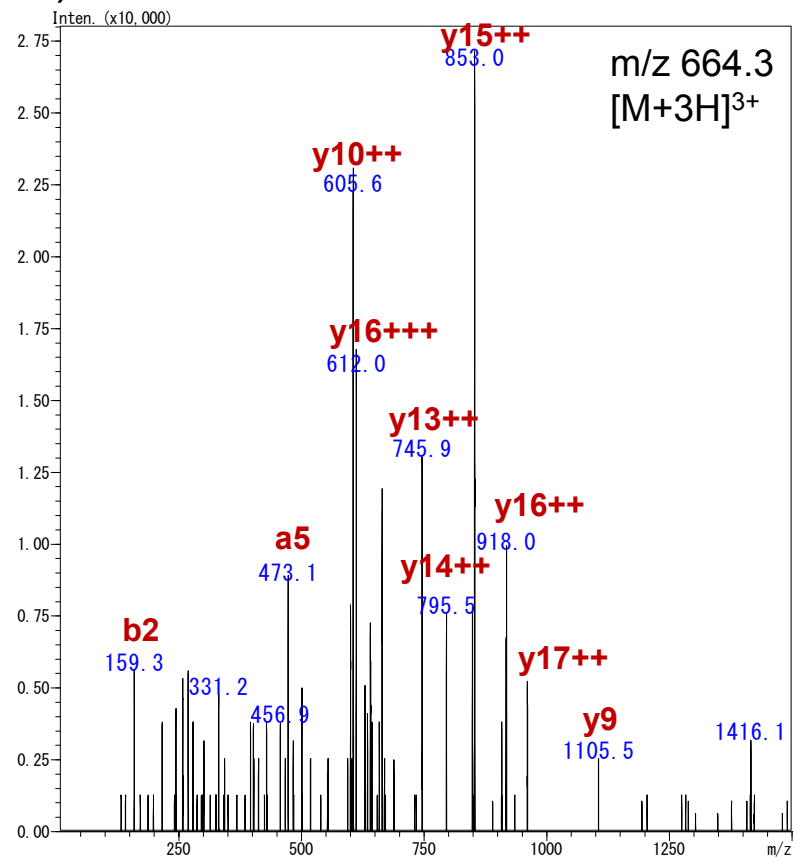


## Supplementary Fig. 3

a) DTYIHWVR

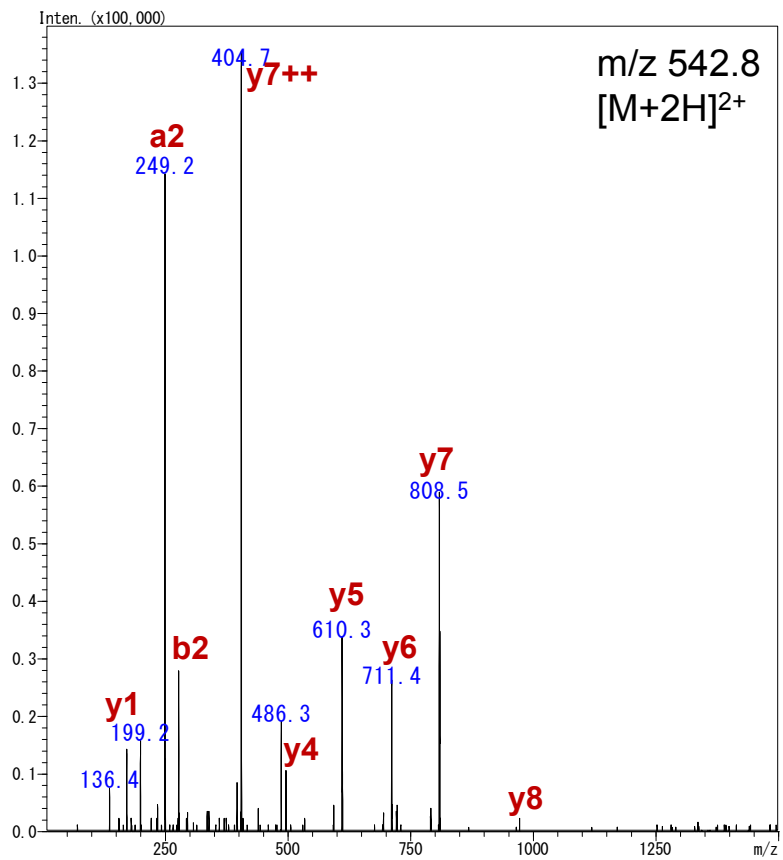


b) ASQDVNTAVAWYQQKPGK



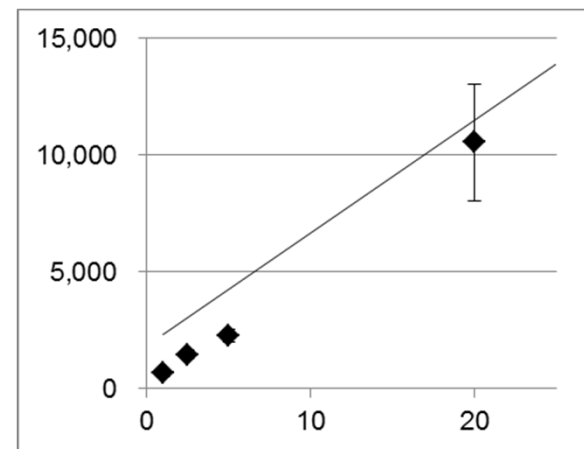
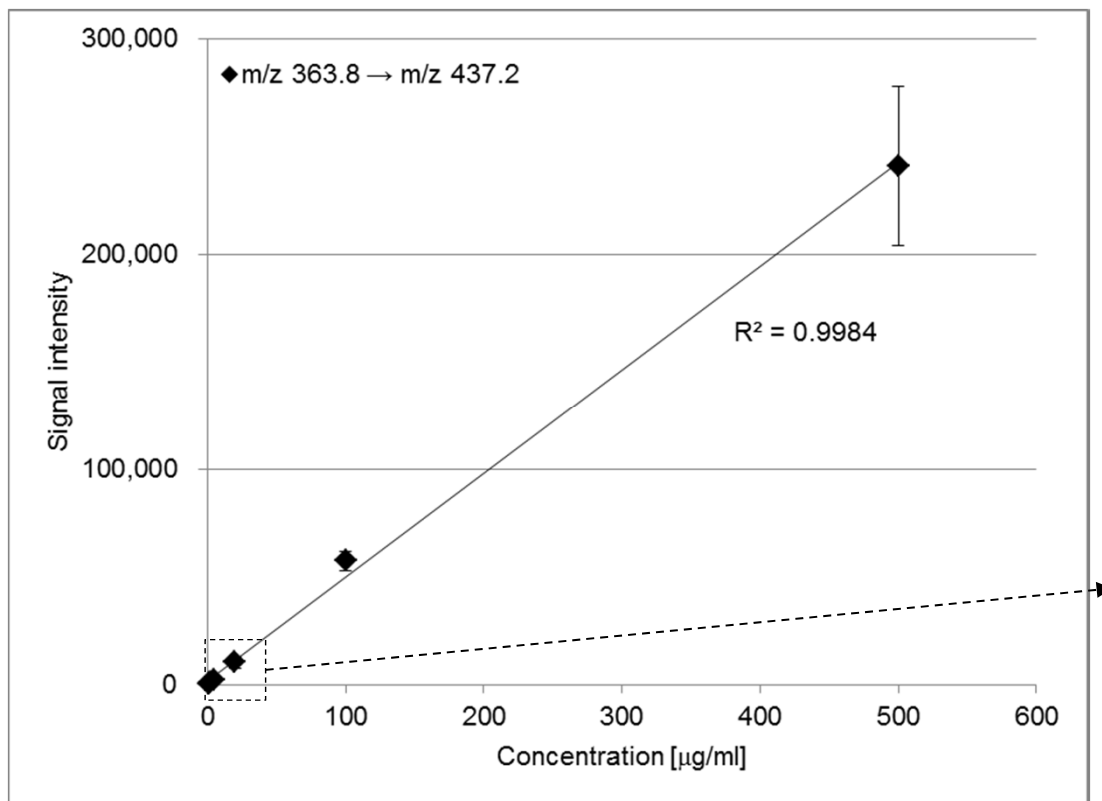
## Supplementary Fig. 3

### c) IYPTNGYTR



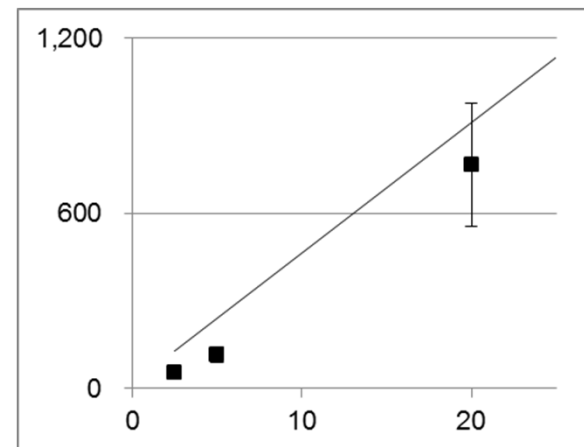
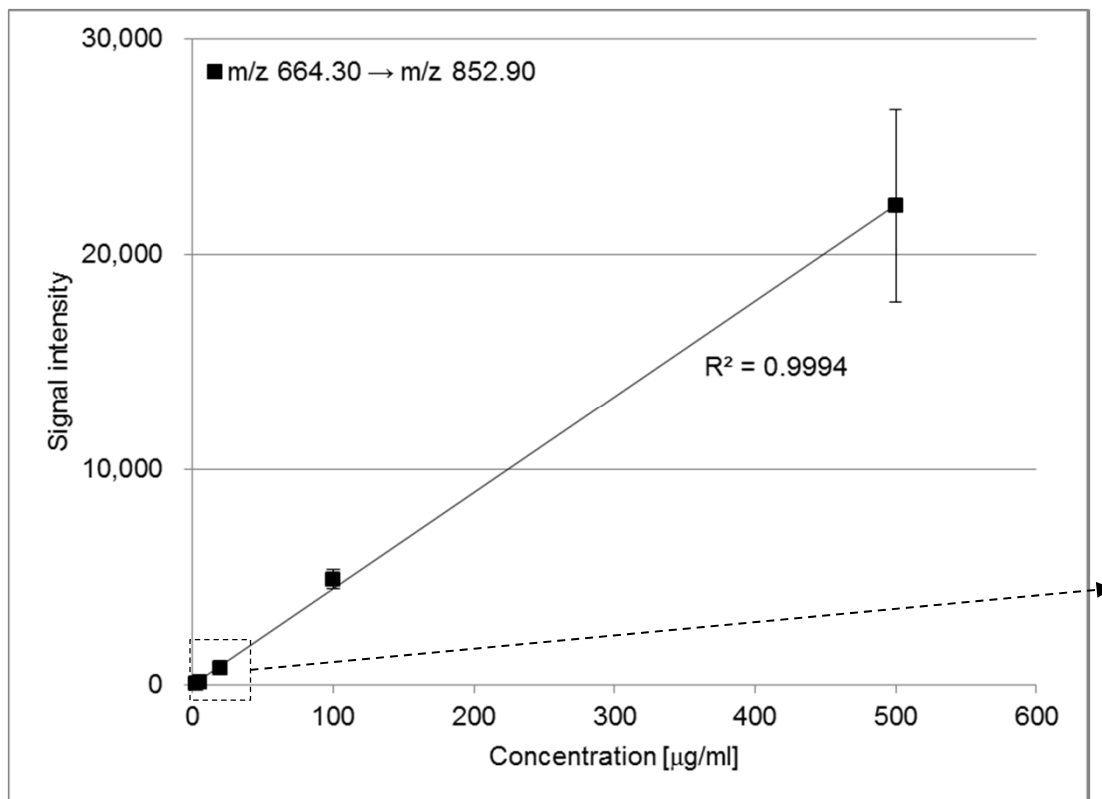
## Supplementary Fig. 4

a)



## Supplementary Fig. 4

b)





## Supplementary Fig. 4

c)

