SUPPLEMENTARY EXPERIMENTAL

Reagents. Peptide standards were synthesized by New England Peptide (Gardner, MA). The purities of the synthetic peptides were >98% as measured by AAA. For stable isotope labeled peptides, all C and N atoms in terminal arginine or lysine were replaced with ^{13}C and ^{15}N. Sequencing-grade trypsin was from Promega (Madison WI). Protease, phosphatase and kinase inhibitor cocktail tablets were from Roche (Basel, Switzerland). Bicinchoninic acid (BCA) protein assay reagents were from Pierce (Rockford, IL). HPLC grade methanol, acetonitrile, acetone, and water were from B&J (Muskegon, MI). LC/MS grade formic acid was from Fluka (Buchs, Switzerland). Tris(2-carboxyethyl)phosphine (TCEP), Tris, iodoacetamide (IAA), and phosphate-buffered saline were obtained from Sigma-Aldrich (St. Louis, MO).

Antibody Production and Purification. The 8c2 cells that secret murine anti-topotecan IgG1 monoclonal antibodies, were grown at 37°C in 1 L spinner flasks containing serum-free media (Hybridoma-SFM, Invitrogen, Grand Island, NY). Following centrifugation and filtration to remove cells and cellular debris, 8C2 was purified by protein-G affinity chromatography via Bio-Rad automated Pressure System. The loading buffer was 20 mM Na2HPO4 (pH 7.0, Sigma Chemical), and the elution buffer was 100 mM glycine (pH 2.8, Sigma Chemical). Eluted antibody was collected in glass tubes containing 1 M Tris buffer to neutralize the solution and minimize antibody aggregation.

The cT84.66 hybridoma cells that produce the monoclonal anti-CEA IgG, were purchased from the American Type Culture Collection (ATCC # HB-8747, Manassas, VA, USA). Antibody was produced and purified using the same procedure as described above for 8c2.

The purity of the antibodies was assessed using sodium dodecyl sulfate polyacrylamide (SDS-PAGE) electrophoresis and the accurate amount/purity of mAb proteins was verified by quantitative amino acid analysis (AAA).

Optimization for Tissue Sample Extraction. Tissue samples from an 8c2-dosed mouse in a pilot study were used for establishing the extraction conditions. The tissue powder was aliquoted into 100 mg fractions, and homogenized in 4 different extraction buffers (a) PBS (100 mM, pH 7.4); (b) PBS with 0.1% SDS; (c) PBS with 0.5% SDS; (d) PBS with 0.1% SDS, 2% NP-40 and 0.5% sodium deoxycholate, respectively. After acetone precipitation and on-pellet digestion, each sample was spiked with IS and subjected to nano-LC/SRM-MS analysis as we described. The extraction performance was evaluated with respect to both extraction recovery and the interferences level. Total protein concentration was determined with the BCA assay.

Identification of SP Candidates Using NanoLC-LTQ/Orbitrap. Peptide separation was performed on an Eksigent two-dimensional nano-LC system (Eksigent Technologies, Dublin, CA) equipped with a nano autosampler. Solvents used were water/0.1% formic acid (mobile phase A) and 85% acetonitrile/0.1% formic acid (mobile phase B). Samples containing 6 μg of peptides were loaded onto a large-ID trap (300 μm ID x 5 mm, packed with Zorbax 3-μm C18 particles) with 3% B at a flow rate of 10 μL/min, and the trap was washed for 3 min. A shallow gradient (flow rate was 250 nL/min) was used to back-flush the trapped samples onto the nano-LC column (75 μm ID x 25 cm, packed with Pepmap 3-μm C18 material): (i) a linear increase from 3% to 10% B over 5 min; (ii) an increase from 10 to 22% B over 55 min; (iii) an increase from 22 to 35% B over 25 min; (iv) an increase from 35 to 60% B over 25 min; (v) an increase from 60 to 97% B over 5 min; and (vi) isocratic flow at 97% B for 15 min. High resolution MS analysis was performed on an LTQ/Orbitrap-ETD hybrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA). MS/MS analysis was performed using a survey scan in FT mode (m/z 350 ~ m/z 1500) with a resolution of 60 000 and an ion accumulation target value of 5×10^6, followed by fragmentation of the 7 most intensive peaks in the linear ion trap. Identification of peptides was performed using BioWorks 3.3.1 embedded with Sequest (ThermoFisher), searching against a FASTA database containing the sequences of target mAbs and murine (or human) proteins derived from the

Swiss-Prot database. The precursor mass tolerance was 10 ppm and the mass tolerance for CID fragments was 1.0 mass unit. Cross correlation score (Xcorr) criteria were ≥4 for 4+ and higher charge states, ≥3 for 3+ ions, ≥2.2 for 2+ ions, and ≥1.7 for 1+ ions. The identified peptides were subsequently filtered by exclusion criteria described in Results and Discussion, and the surviving peptides were designated "candidate peptides" (*cf.* Supplementary Table S-1).

Peptide Stability Test. mAb standards were spiked into the extraction of a target tissue at 5 μg/mL each and digested as described above, and then the mixture was used for stability assessment. Peptide stabilities were evaluated by incubation under the conditions of (i) tryptic digestion (37 °C, pH) 8.5) and (ii) storage in the autosampler (4°C, pH2.8). For evaluation of stability under digestion conditions, the solution was sampled at 0, 1, 2, 4, 8, 12, and 18 h after the completion of digestion. At each time point, a 20 μL sample was taken, acidified by adding formic acid to a final concentration of 1% (v/v), and then analyzed immediately by nano-LC/MS using the optimized conditions obtained in the OAO procedure. For assessment of candidate stability under autosampler storage conditions, the digestion mixture was acidified immediately upon completion of digestion, incubated in the refrigerated autosampler, and analyzed at 0, 1, 2, 4, 8, 12, 24 and 48 h. Any candidate that degraded more than 20% within either evaluation period was disqualified.

Plasma Sample Analysis. The plasma samples were allowed to thaw at room temperature. After vortex-mixing, an aliquot (2 μL) of plasma were diluted with PBS by 10 times. Then an acetone precipitation/on-pellet digestion procedure was applied as described in the Experimental. The same nano-LC/SRM-MS method was employed to analyze plasma sample. The established quantification range was 1.56 ~ 780 μg/mL. The plasma concentrations were shown in Supplementary Figure S-4.

Calibrations and Method Evaluation. Calibrators were prepared for each tissue matrix, by spiking various amounts of mAb into aliquots (50μL) of blank tissue extracts. The final concentrations were 0.156, 0.312, 0.780, 1.56, 3.12, 7.80, 15.6, 31.2 and 78.0 μg/g tissue for 8c2 in brain, liver, heart, kidney;

0.312, 0.780, 1.56, 3.12, 7.80, 15.6, 31.2, 78.0 and 155.9 μg/g tissue for 8c2 in spleen and lung; 0.307, 0.767, 1.53, 3.07, 7.67, 15.3, 30.7, 76.7 and 153 μg/g tissue for cT84.66 in xenograft tumor; All spiked solutions were vortexed, incubated at 4 °C for overnight and then precipitated, digested and analyzed by nano-LC/SRM-MS. Isotopic-labeled I.S. peptides were spiked into the samples prior to digestion at the concentration of 40.0 μg/g tissue (calculated as mAb protein concentration).

Calibration curves were constructed by plotting the peak area ratios of an SP and its I.S. versus the corresponding mAb concentrations and linear regression with a $1/x^2$ weighting factor was employed. Method accuracy and precision were evaluated during the three validation runs using quality control (QC) samples prepared by spiking mAb standard into blank matrix at three different levels (0.390, 7.80 and 70.2 μg/g tissue for 8c2 in brain, liver, heart, kidney; 0.390, 7.80 and 125 μg/g for 8c2 in spleen and lung; 0.384, 7.67 and 123 μg/g for cT84.66 in xenograft tumor). The investigations of extraction efficiency and peptide stability were described in Supplementary Experimental.

For the batch-wise analysis of tissue samples, all quantitative data were confirmed using two criteria: i) the discrepancy of protein concentrations determined independently from the two SPs is within 25% of the higher value; ii) the ratio variation of quantification and qualification transitions was within the accepted tolerance: ±25% for transitions with the same precursor ion compared to standard runs.

Comparison of Calibration Curves for MAb Using Protein Standards vs Synthesized Peptides. Blank brain tissue was homogenized and extracted. A 50-μL aliquot was subjected to acetone precipitation/on-pellet digestion as described. Peptide calibration curves were prepared by spiking the mixed solutions of two SPs for 8c2 (A_{67}TIITDTSSNK$_{77}$ and T_{156}LADGVPSR$_{164}$) into the blank tissue digests at concentrations of 0.156, 0.312, 0.780, 1.56, 3.12, 7.80, 15.6, 31.2 and 78.0 μg/g tissue (calculated by protein conc.). The same nano-LC/SRM-MS method was used to establish linearity as described above. To access the accuracy, purified 8c2 protein was spiked into aliquots of blank brain

extracts at 0.390, 7.80 and 70.2 μg/g tissue and analyzed using the calibration curves constructed with synthesized peptides. The same set of spiked samples was analyzed in parallel using calibration curves constructed using 8c2 protein standard.

SUPPLEMENTARY RESULT AND DISCUSSION

Assessment of the stability of SP candidates. Degradation/modification of SP may severely compromise the accuracy, sensitivity and reproducibility of protein quantification [22]. Such risks are not readily predictable from the peptide sequence. Therefore, it is necessary to experimentally examine the peptide stability in the digested target matrices prior to SP selection. Using the nano-LC/SRM-MS methods developed by the OAO procedure, such evaluation can be easily performed. Two sets of spiked target matrix samples (*e.g.* pooled brain homogenates) were subjected to the precipitation/on-pellet-digestion procedure, and then used for stability evaluation. Upon the completion of the digestion, the tryptic peptide mixtures were further incubated in either (i) digestion buffer at 37°C for up to 18 h or (ii) in the injection solution (*i.e.* the digested buffer acidified with 1% formic acid) at 4°C for up to 48h, respectively mimicking the environments to which the tissue digests are exposed during tryptic digestion or queuing in a cooled autosampler. Subsequent LC/MS measurements revealed that 2 and 3 candidate peptides respectively for 8c2 and cT84.66 were instable in brain digest mix after incubation at 37°C for 18h (Supplementary Fig S-2). Additionally, 5 and 6 peptides respectively from 8c2 and cT84.66 fell out of the acceptable range (decrease of signal < 20%) after sitting under autosampler conditions for 48h (Supplementary Fig S-3). These unstable candidates were disqualified. The stability plots in most tissue specimens showed similar trends with an exception of the liver, where one additional peptide (S_{296}VSELPIMHQDWLNGK$_{311}$) appeared to be unstable (37% signal loss) after incubation at 37°C for 18 h.

Further investigation revealed that the quantitative biases introduced by these unstable peptides cannot be compensated for by the addition of isotopic-labeled peptides prior to digestion (data not shown), probably due to the delayed decay of the newly-released peptides from protein compared to the spiked IS [20, 22]. While the study of degradation mechanisms of unstable peptides is beyond the scope of this work, the results suggested the considerable prevalence of unstable tryptic peptides, which could

severely compromise the quantitative accuracy, sensitivity and reproducibility if selected as the SP. Furthermore, among the unstable peptides, some exhibited high LC/MS response, which underscores the risk of selecting SP merely based on the sensitivity achieved.

SUPPLEMENTARY TABLES AND FIGURES

SI Table 1 (a) Candidate peptide list of 8c2 identified by LTQ-Orbitrap [a, b]

Peptides	Abbreviation	Charge state	Precursor m/z	Top 5 most intensive product ions	RT (min)
Light chain					
F_{165}SGSGSGTQYSLK$_{177}$	FK13	2+	659.8	651.0; 642.1; 796.4; 940.5; 1084.5	22.3
S_{146}PQLLVYNAK$_{155}$	SK10	1+	1132.6	1115.6; 986.5; 638.5; 495.4; 783.4	40.5
		2+	566.8	707.5; 594.3; 495.3; 820.5; 539.3	40.5
T_{156}LADGVPSR$_{164}$	TR09	1+	915.5	515.3; 871.5; 897.5; 342.3; 359.4	48.9
		2+	458.3	701.4; 359.3; 557.3; 215.1; 630.4	48.9
A_{128}SGNIHNSLAWYQQIK$_{143}$	AK16	2+	915.5	906.8; 679.4; 1250.7; 936.5; 1136.6	64.8
		3+	610.7	842.7; 679.4; 576.4; 433.4; 865.5	64.8
Heavy chain					
E_{287}EQFNSTFR$_{295}$	ER09	2+	579.3	510.3; 624.4; 771.4; 561.5; 423.4	27.5
A_{339}PQVYTIPPPK$_{349}$	APK11	2+	605.9	438.3; 570.5; 815.5; 914.5; 652.5	30.8
V_{253}TCVVVDISK$_{262}$	VK10	2+	560.3	919.5; 542.4; 660.4; 759.6; 460.2	35.7
V_{317}NSAAFPAPIEK$_{328}$	VK12	1+	1243.7	654.4; 486.3; 801.5; 872.6; 1226.7	38.9
		2+	622.3	613.5; 654.4; 801.5; 872.5; 590.3	38.9
		3+	415.2	327.7; 654.5; 443.4; 486.4;572.4	38.9
S_{409}NWEAGNTFTCSVLHEGLHNHHTEK$_{433}$	SK25	3+	969.1	1195.4; 1159.9; 902.5; 1353.0; 958.1	53.7
		4+	727.1	902.3; 840.4; 797.3; 773.4; 714.2	53.7
A_{67}TIITDTSSNK$_{77}$	AK11	2+	575.8	567.0; 651.3; 752.4; 865.4; 978.5	62.5
D_{243}VLTITLTPK$_{252}$	DK10	1+	1100.7	1082.6; 708.4; 821.4; 559.4; 985.6	84.9
		2+	550.8	541.9; 345.3; 886.6; 756.5; 738.5	84.9
N_{387}TQPIMDTDGSYFVYSK$_{403}$	NK17	2+	983.5	1622.8; 1281.6; 1412.5; 876.2; 1166.6	90.4
		3+	850.1	1103.3; 838.4; 985.4; 767.0; 1167.4	90.4
S_{296}VSELPIMHQDWLNGK$_{311}$	SK16	2+	927.5	1338.6; 726.7; 834.7; 997.4; 670.1	94.7
		3+	618.7	834.6; 670.1; 726.3; 550.8; 790.9	94.7
T_{120}TPPSVYPLAPGSAAQTNSMVTLGCLVK$_{147}$	TK28	2+	1430.8	1329.6; 1833.9; 1905.0; 1139.8; 1009.5	98.2
		3+	954.2	1358.1; 1171.9; 917.9; 1027.6; 516.3	98.2
D_{263}DPEVQFSWFVDDVEVHTAQTKPR$_{286}$	DR24	2+	1423.2	1308.4; 938.6; 1365.9; 1380.6; 801.4	106.4
		3+	949.1	872.8; 1145.9; 1081.8; 1008.2; 556.2	106.4
		4+	712.1	872.7; 748.5; 629.4; 556.2; 1173.2	106.4

[a] The uniqueness for 8c2 was confirmed by searching against a murine database with BLAST;

[b] Peptides were ordered according to their LC elution profiles using a shallow gradient (Supplemental Experimental).

SI Table 1 (b) Candidate peptide list of cT84.66 identified by LTQ-Orbitrap [a, b]

Peptides	Abbreviation	Charge state	Precursor m/z	Top 5 most intensive product ions	RT (min)
Light chain					
F_{86}SGTGSR_{92}	FR07	2+	356.2	347.1; 477.2; 267.2; 235.1; 207.2	18.1
V_{174}DNALQSGNSQESVTEQDSK_{193}	VK20	2+	1068.5	707.4; 1495.6; 893.3; 1051.7; 1902.9	22.4
		3+	712.7	748.6; 698.0; 812.6; 641.3; 606.2	22.4
V_{215}YACEVTHQGLR$_{226}$	VR12	2+	716.9	586.1; 810.5; 667.6; 1170.6; 610.5	27.8
		3+	478.2	586.1; 235.2; 668.2; 550.5; 263.3	27.8
A_{75}SNLESGIPVR$_{86}$	AR11	1+	1142.6	628.4; 1124.6; 371.4; 541.4; 870.6	39.4
		2+	571.8	371.4; 757.5; 772.3; 628.6; 541.5	39.4
T_{133}VAAPSVFIFPPSDEQLK$_{150}$	TK18	2+	973.5	913.4; 802.7; 1015.5; 1060.5; 1320.6	68.7
		3+	649.3	457.5; 913.4; 873.5; 517.3; 1060.7	68.7
A_{45}GESVDIFGVGFLHWYQQK$_{69}$	AK25	3+	727.7	869.7; 962.8; 919.2; 812.3; 575.6	103.8
Heavy chain					
I_{70}DPANGNSK$_{78}$	IK09	1+	915.5	687.5; 670.4; 898.5; 405.2; 519.4	10.6
		2+	458.2	687.5; 344.5; 229.2; 255.1; 519.4	10.6
A_{350}LPAPIEK$_{357}$	AK08	1+	838.5	654.3; 486.3; 563.3; 340.2; 508.3	27.5
D_{272}TLMISR$_{278}$	DR07	1+	835.4	720.4; 461.2; 619.4; 506.3; 574.2	30.6
		2+	418.2	409.4; 506.3; 619.4; 375.2; 262.1	30.6
D_{50}TYMHWVK$_{57}$	DK08	1+	1079.5	933.4; 1061.4; 834.4; 964.6; 648.2	32.4
		2+	540.3	531.4; 432.3; 482.7; 700.3; 863.6	32.4
L_{39}SCTASGFNIK$_{49}$	LK11	1+	1197.6	1179.7; 1067.5; 1051.5; 665.5; 578.4	42.6
		2+	599.3	499.6; 665.5; 837.5; 736.5; 578.4	42.6
F_{298}NWYVDGVEVHNAK$_{311}$	FK14	1+	1677.8	1678.7; 853.4; 1659.8; 1531.6; 1460.7	43.7
		2+	839.4	968.5; 853.3; 1067.5; 1230.6; 697.4	43.7
		3+	559.9	709.1; 534.6; 615.8; 485.0; 262.1	43.7
Q_{58}RPEQGLEWIGR$_{69}$	QR12	2+	734.9	726.4; 531.4; 921.5; 1184.6; 345.2	52.3
		3+	490.3	480.2; 612.2; 562.6; 660.4; 830.5	52.3
G_{394}FYPSDIAVEWESNGQPENNYK$_{415}$	GK22	2+	1272.6	764.4; 1150.6; 1465.6; 1780.7; 1764.8	62.2
		3+	848.7	798.2; 1089.3; 950.5; 764.4; 733.5	62.2
G_{145}PSVFPLAPSSK$_{156}$	GK12	1+	1186.6	699.4; 1168.6; 751.4; 769.4; 846.5	64.6
		2+	593.8	699.4; 846.5; 769.4; 418.2; 470.2	64.6
N_{384}QVSLTCLVK$_{393}$	NK10	2+	552.8	916.6; 820.5; 342.1; 519.5; 572.1	71.8
S_{157}TSGGTAALGCLVK$_{170}$	SK14	1+	1264.7	1321.7; 1303.6; 576.6; 1133.7; 760.5	76.7
		2+	632.8	652.4; 576.3; 689.5; 760.5; 831.4	76.7
T_{279}PEVTCVVVDVSHEDPEVK$_{297}$	TK19	2+	1041.5	1667.8; 472.2; 1039.5; 1154.5; 1253.6	86.1
		3+	694.7	834.6; 472.2; 971.2; 680.3; 856.8	86.1
V_{325}VSVLTVLHQDWLNGK$_{340}$	VK16	2+	904.5	997.5; 1110.5; 896.7; 617.2; 1192.6	94.8
		3+	603.3	806.1; 805.4; 712.6; 656.1; 499.5	94.8

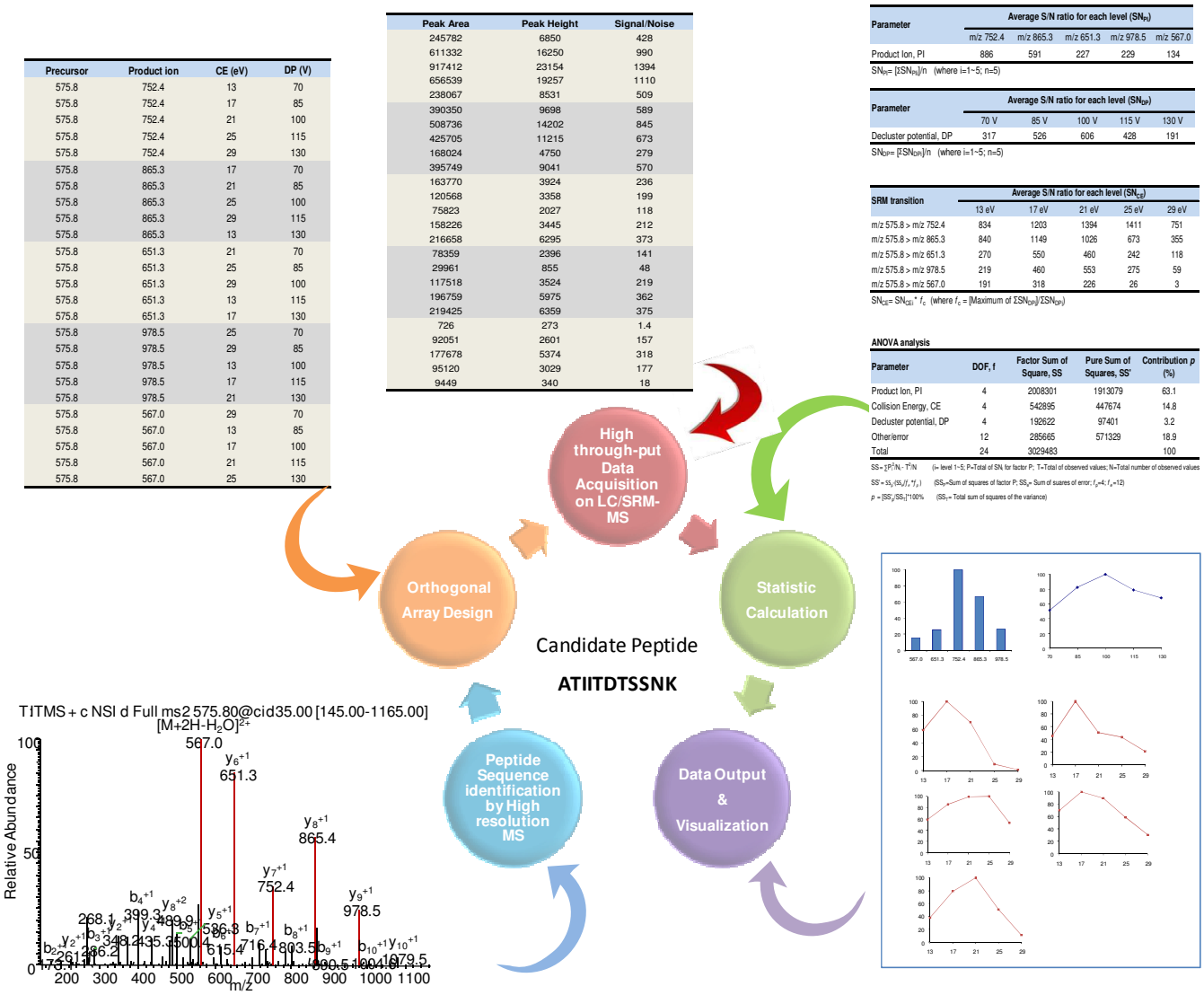
[a] The uniqueness for cT84.66 was confirmed by searching against a human database with BLAST;

[b] Peptides were ordered according to their LC elution profiles using a shallow gradient (Supplemental Experimental).

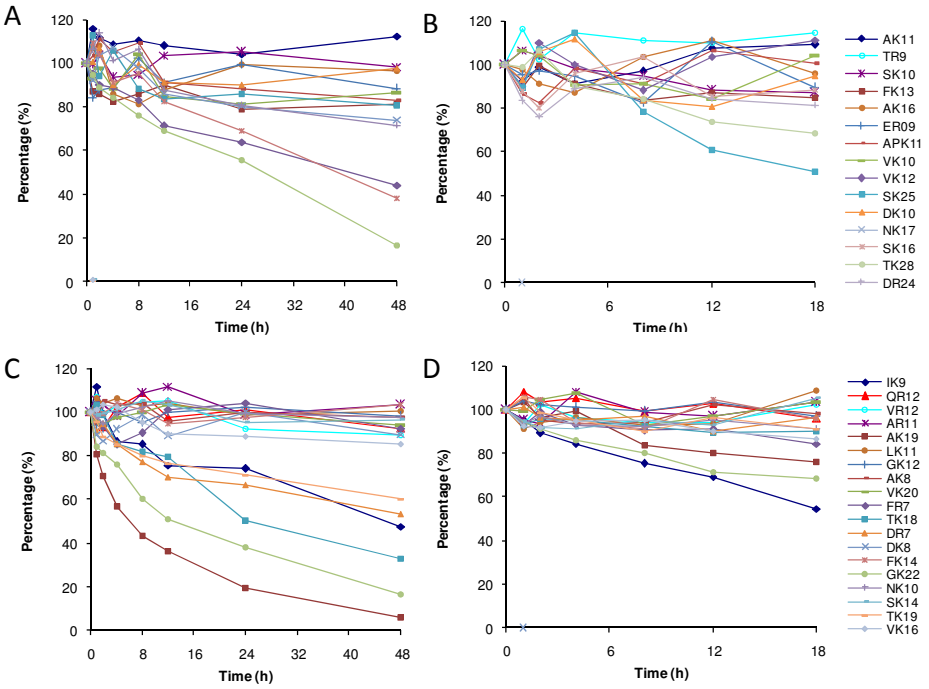
SI Table 2. Accuracy resultes caculated by protein calibration curve and peptide calibration curve

QC level (pmol/g) [a]	Accuracy (RE, %)			
	Protein calibration (TR09)	Protein calibration (AK11)	Peptide calibration (TR09)	Peptide calibration (AK11)
2.5	10.6	-4.3	-51.8	-27.9
50	-8.8	-2.7	-46.0	-31.1
450	-3.2	9.3	-50.3	-25.2

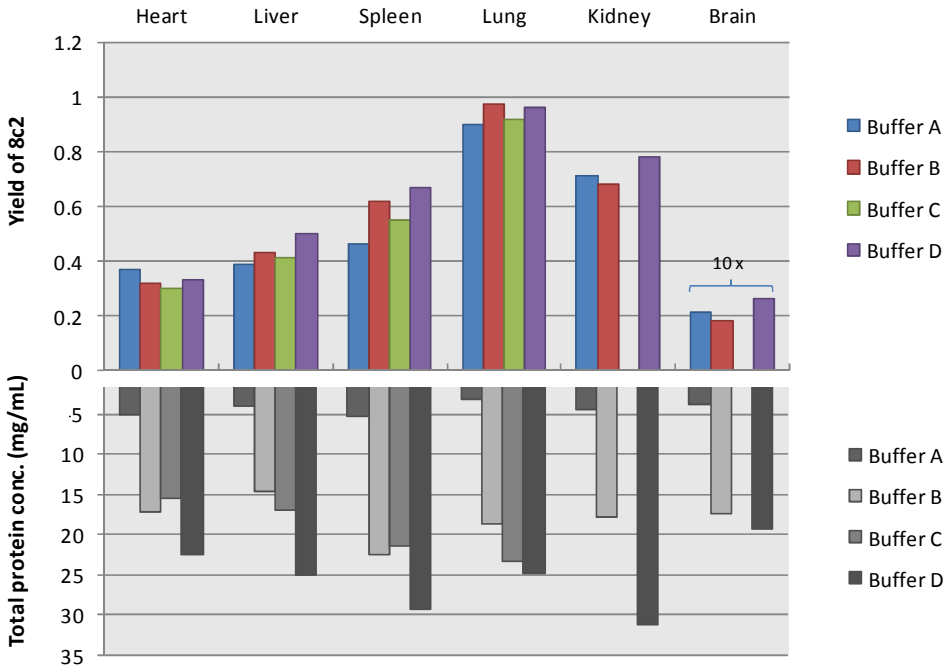
[a] corresponds to 8c2 concentration of 0.390, 7.80 and 70.2 μg/g, respectively



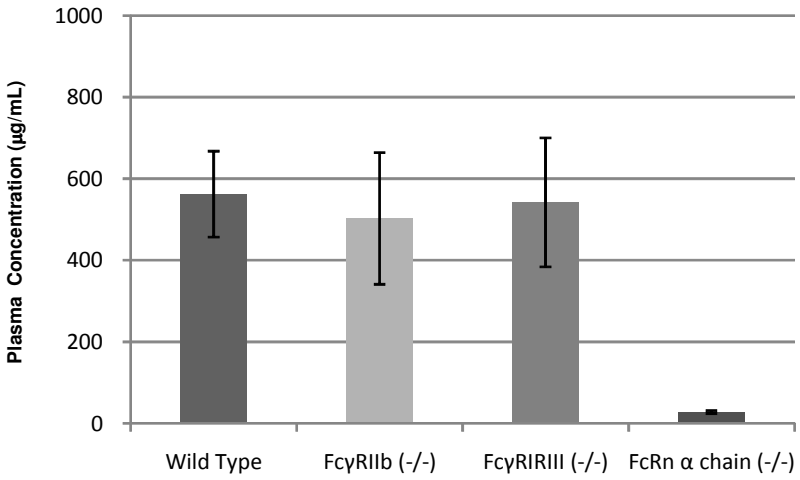
Supplementary Figure S-1 Illustration of OAO method setup and data processing using the optimization of a candidate peptide, A_{67}TIITDTSSNK$_{77}$, as the example.



Supplementary Figure S-2 Time courses of the stability evaluation for the two candidate peptides derived from 8c2 at (A) 4 °C and pH2.5 for 48 h and (B) 37 °C and pH8.2 for 18 h, and from cT84.66 at (C) 4 °C and pH2.5 for 48 h and (D) 37 °C and pH8.2 for 18 h. Peptide abbreviations are shown in Supplementary Table S1.



Supplementary Figure S-3 Comparison of the extraction efficiency by different extraction buffers for tissues acquired from a dosed-mouse in a pilot study. The absolute yield for 8c2 extraction was expressed as the peak area ratio of signature peptide and IS peptide. The concentration of total proteins in the extracts was measured using BCA method. Buffer C was not used in kidney and brain extraction due to the limited tissue amount (< 400 mg). The brain data were exaggerated by 10-fold for better visualization. Buffer A: 100 mM PBS (pH 7.4); Buffer B: PBS with 0.1% SDS; Buffer C: PBS with 0.5% SDS; Buffer D: PBS with 0.1% SDS, 2% NP-40 and 0.5% sodium deoxycholate.



Supplementary Figure S-4 8c2 concentrations in the plasma of wild type, FcRn(-/-), FcγRIIb(-/-) and FcγRI/RIII(-/-) mice after intraperitoneal injection 8c2 at 1 mg/kg for 73 days. Data shown as Mean ± SD (n = 6 per group). The plasma sample processing and analysis were described in Supplementary Experimental.