Supporting information

High-throughput, sensitive quantification of protein therapeutics and biomarkers using an antibody-free, peptide-level multiple-mechanism enrichment via strategically regulated pH, ionic and organic solvent strengths

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Supplemental Experimental

Reagents

HPLC-grade methanol, acetonitrile, acetone, and water were purchased from B&J (MI, USA). Formic acid was purchased from Fluka (Buchs, Switzerland). Sodium dodecyl sulfate (SDS) was purchased from G-Biosciences (MO, USA). Ultra-pure grade Tris was purchased from MP Biomedicals (OH, USA). Dithiotheritol (DTT) was from GE healthcare (Uppsala, Sweden). Iodoacetamide (IAA) and trypsin were purchased from Sigma-Aldrich (MO, USA). Bicinchoninic Acid Protein Assay (BCA) kit was from Thermo Fisher Scientific (IL, USA). The following standard proteins: CD3 and IL-33 were purchased from Sino biological (Shanghai, China) and then purified. T84.66 was produced and purified in Dr. Joseph Balthasar lab. AB095 was donated by AbbVie. Anti-mCD3 was donated by Janssen. Stable-isotope-labeled-peptide internal standard (SIL-peptide I.S.) with K[15N, 13C] or R[15N, 13C] at C-terminal for each SP was obtained from Synpeptide (Shanghai, China).

Tissue sample homogenization and extraction

The frozen tissues were dissected and ground into fine powder in the presence of liquid nitrogen. An aliquot of 50.0 mg of tissue powder (or keep the same tissue powder and buffer ratio) was suspended in 500 µL of lysis buffer (50 mM Tris, 150 mM NaCl, 0.5% sodium deoxycholate, 2% SDS, 2% NP-40, pH was adjusted to 8.0 by formic acid) and homogenized in ice bath using a Polytron homogenizer (Kinematica, Lucerne, Switzerland). After sonication for 30s, the homogenates were centrifuged at 20000×g for 30 minutes at 4°C, and the supernatants were isolated and stored at -80 °C.

Surfactant aided precipitation/on-pellet digestion (SOD) protocol

SOD digestion protocol has been modified from previous practiced and reported protocol[1]. SDS in phosphate buffered saline (PBS) is spiked to plasma or tissue extract sample containing final SDS concentration of 1%, the total protein concentration was adjusted to 2 µg/µL; pH of the solution is 8.0. Dithiothreitol (DTT) is added at 10 mM followed by incubation under 56 °C for 30 minutes to reduce the proteins, and then iodoacetamide (IAA) was added at 25 mM and incubated in the dark for 30 minutes at 37 °C. One volume of chilled acetone was added so that the mixture turned cloudy but no visible particulate was observed; another 5 volumes of chilled acetone was then added step-wise with vigorous vortexing, followed by 3-hour incubation at -20°C. After centrifugation at 20000×g under 4°C for 30 minutes, the supernatant was poured out, washed again with 1 volume of acetone:water 6:1 (v/v) and the pellet was allowed to air dry. Tris buffer with trypsin (50 mM, pH=8.5) was added to the pellet at an enzyme-to-substrate (E/S) ratio of 1:25 (w/w) and incubated for 45 min with vigorous vortexing at 500 rpm and 37 °C in an Eppendorf Thermomixer, which completely dissolves the pellet and to achieve complete digestion.

Trapping micro LC-MS (T-µLC-MS)

The method was adopted based on a previous reported protocol[2]. The system contained an UltiMate 3000 LC system (containing SRD-3400 degaser, NCS-3500RS CAP pumps and a high-flow binary gradient pump, and WPS-3000TBRS autosampler with a 250-µL loop) coupled to a TSQ Altis triple-quadrupole mass spectrometer via an Ion Max NG ion source with H-ESI probe and 34-G narrow-bore spray needle (Thermo Fisher Scientific, CA, USA). Sample trapping is conducted on a C8 column (15 × 2.1mm, 3.5-µm particle size, 100 Å, Agilent, CA, USA) at flow rate of 1 mL/min using the high-flow binary pump. High-flow loading mobile phase (MP) $A_{trapping}$ and $B_{trapping}$ were water: acetonitrile of 98:2 and 5:95 (v/v) containing 1mM ammonium

formate (pH=9.0, adjusted by ammonium hydroxide), respectively. A micro flow selector (5-50μL/min) was used for μLC-MS. The separation column was a Xselect CSH C18 column (150× 0.5 mm, 2.5 μm, 130 Å, customized packed) at a flow rate of 25 μL/min. Low-flow MP $A_{analysis}$ and $B_{analysis}$ for μLC were water:acetonitrile:formic acid of 98:2:0.1 and 15:85:0.1 (v/v/v, pH=3.0). At the beginning of sample delivery from trap to column, a 1-min isocratic elution with the initial gradient $B_{analysis}$ % was used to help peak compression. A ZDV 6-port valve placed in the heated column compartment was utilized to coordinate operation of the two flow systems. The separation temperature was controlled at 40°C. The Spray voltage was 3.5 kV, vaporizer temperature was 50°C, Sheath gas was 15.0 Arb, Aux gas was 2.0 Arb, and the capillary temperature was maintained at 325°C. The optimized RF-lens voltages and collision energies were obtained for each signature peptide by on-the-fly orthogonal array optimization (OAO) strategy[3,4]. SRM transitions and conditions of the targets are listed in Table S1. Isolation window was set to 0.2 Th for Q1, and 0.7 Th for Q3 for all channels.

Preparation of calibration curve and validation

Standard proteins were spiked into blank plasma or tissue homogenates to prepare calibration curves with concentration range as shown in Table **2**. The SP of human IL-33 is unique in mouse proteome based on protein blast, and there was no detectable signal for it in the blank mouse plasma based on our pilot experiment. Rat plasma was used as a surrogate matrix for validation of CD3 (SP is unique in rat proteome, based on protein blast and pilot LC-MS results). Mice colon tissue was utilized for validation of quantification method of CEA, which had no detectable signal for SP of CEA (data not shown). Hybrid calibration strategy was used in this study as described previously[5]. All the samples were prepared by optimal CX-RP enrichment procedure. Calibration curves were established by plotting extracted ion current peak area ratio

of the target/IS as a function of analyte concentration, weighted least-squares linear regression analysis of the standard curves was used. A batch of quality control (QC) samples were prepared containing low, medium, and high concentrations of target protein in biomatrices, which was compared to independently prepared calibration curves. Precision of the assay was calculated by repeated analysis of QC samples, and the coefficient of variation (CV%) of the replicate measurements was calculated to determine variability. Quantitative accuracies of the QC samples at each concentration level were calculated against nominated concentrations.

Matrix effect.

To evaluate the matrix effect after enrichment, digest of blank matrix was aliquoted and enriched (***Antibody-free, multiple-mechanism enrichment***) with optimal conditions for each target (Table 1) and generic MCX SPE (***Wash/elution condition optimization section***), respectively. And then SP was spiked into each reconstituted supernatant to 10 ng/ml accordingly. Each SP was also spiked into Tris buffer (50mM, containing 1% formic acid) to 10 ng/ml in parallel. Relative high concentration of SP was used in this experiment to counteract the possible nonspecific binding in neat solution and remained interferences after generic MCX SPE. The ion suppression was calculated by the following equation:

$$Ion\ suppression = \frac{Response_{neat\ solution} - Response_{Enriched\ sample}}{Response_{neat\ solution}}.$$

Animal study

1 x10^7 $MC38^{CEA+}$ cells were injected subcutaneously to C57BL/6 male mice at right flank separately. Treatments started when the tumor size reached 300 mm^3. 0.5 mg/kg T84.66 were administered intravenously through penile vein. Blood samples (0.20 ml) were collected before

dosing, and 1, 3, 8, 24, 72, 168, 216 and 336 hours post administration in heparin-treated tubes (n=3 per time point). All blood samples were centrifuged at 2000×g for 10 min to isolate the plasma and stored at -80 °C until assay. Tissue samples (heart, liver, spleen, lung, kidney, tumor, brain, colon, and muscle) were collected after transcardial perfusion at each time point. The excised tissues were blotted dry with a lint-free tissue, snap-freezed with liquid nitrogen, and stored at -80 °C until assay.

Supplemental Results and Discussion

Several important points during the CX-RP method development are summarized here:

1) CX-RP sorbents from different vendors were evaluated carefully for selective and efficient peptide separation; surprisingly, these products showed remarkable differences in terms of separation efficiency, recovery, robustness and reproducibility. In pilot study, we observed the Strata-X-C cartridge was markedly superior for this particular application, owing to the considerably higher peptide recovery and excellent reproducibility in peptide separation (detailed data not shown).

2) Prior to peptide separation, we found that it is critical to condition the sorbent with 100% methanol and ***then 2% formic acid***, in this sequence prior to sample loading. After loading, sequential washes with 2% formic acid and 100% methanol (in this order) are conducted to effectively remove non-positively charged matrix components (some conjugate-base of salts, lipids, fatty acids, *etc.*) and thus "purifying" the peptides. These steps were found to greatly improve consistency and reduce various artifacts in following selective washing and elution steps.

3) To ensure the retention behaviors of peptides can be precisely controlled during CX- and RP-wash steps, we segregate the two mechanisms in either step, by optimizing the strength of the intended desorption mechanism while keeping the retention by the other mechanism at a constant and reduced level. For example, when performing the CX-wash, a relatively high, experimentally identified (discussed below) organic solvent strength is maintained to weaken hydrophobic retention and facilitate precise identification of an appropriate salt concentration to achieve maximal selectivity. Similarly, we suppressed the ionic retention for RP-wash.

4) Our preliminary study showed the loading capacity under CX-mechanism was much higher than RP-mechanism (data not shown); therefore, we performed CX-wash before RP-wash to permit high loading capacity for biological samples.

5) As shown in **Figure 1a**, both CX- and RP- retention behaviors of a peptide is profoundly affected by the pH relative to pI. Consequently, precisely regulated pH is critical to achieve high selectivity. After examination of a number of buffer systems, we found buffer salt listed in **Table S3** afforded us great success for peptide separation. It is important to note pH

adjustment is compounded by CX sorbent which ***may change buffer pH substantially*** when an inappropriate buffer system is used. For example, the pH of 1x PBS buffer (20 mM) can drop 6 units after flowing thru the cartridge. Furthermore, the buffer concentrations should be high enough (*e.g.*, at least 50 mM) to enable reproducible separation. Finally, we found using an organic-solvent-compatible pH meter is important to accurately adjust pH in the water/methanol mixture.

6) For optimization of each washing and elution step, the optimal conditions are these achieving the highest Target Enrichment Ratio (TER, defined as the normalized ratio of the relative recovery of target SP over the mean relative recovery of the 40 selected matrix peptides) AND at least 75% recovery of the target SP. The selectivity evaluated by these criteria has been verified by S/N (data not shown).

7) Although inorganic salts were employed for selective CX- and RP-washes for reasons discussed above, in the selective elution step, organic salts are used along with an optimized, relatively high level of organic solvent, to permit facile removal of these components before LC-MS analysis.

8) During method development, it is important to fine-tune the organic solvent % owing to the observed exponential dependence of peptide desorption on organic solvent % (**Figure 1b)**, regardless of pH or ionic strength as long as these are kept constant. Conversely, as discussed above, the desorption effect by salt concentrations can be linearly scaled when identifying the optimal salt concentrations for CX-wash.

9) For method development, the synthesized target SP is spiked in the digested tissue or plasma samples before CX-RP enrichment and SIL-peptide-IS is spiked after enrichment to measure recovery.

10) Finally, though SPE is considered a chromatography-based approach, it appears the continuous desorption profile (*e.g.*, **Figure 1b and 1c)** for peptides is quite different from that by liquid chromatography[2], most likely because SPE separation is based on a static equilibrium in a short cartridge rather than directional migration of compounds as on a column. It is important to keep this feature in mind during method development.

Quantitative method development

Methods for quantification of the 6 proteins plasma/tissues have been established. No interference was found in blank matrices for the SP of the three mAb and the three drug-targets/biomarkers (CD3, IL30 and CEA) in the blank matrix or surrogate blank matrix (**SI Experimental Section)**, indicating high selectivity. To prevent the risk of severe negative biases when using synthesized SP as calibrator[5], we employed purified mAb and target proteins, with purities accurately measured by quantitative amino acid analysis (AAA), to prepare the calibration curves. All proteins showed excellent linearity over at least 500-fold concentration ranges, as shown in **Table 2**. **Table 2** also showed excellent LOQs (2-45 ng/mL plasma or ng/g tissue) for the 6 proteins, a great improvement (up to 20 folds) over a generic MCX SPE. We speculate such level of LOQs are sufficient for PK/PD investigation of the vast majority of mAb and targets. The accuracy and precision of 6 proteins ranged from 85.7% to 103.4% and 5.7% to 16.1%, respectively (**Table 2**), which indicated that the overall sample preparation, CX-RP enrichment and analytical procedure are quantitative and reproducible. Additionally, we observed the quantitative methods developed here are not matrix selective, *i.e.*, they work in various tissues and plasma, likely attributed to the extensively simplified matrix by the CX-RP procedure.

Supplemental Tables

Table S1. Model proteins, matrices and signature peptides (SP)

Protein name	Type	Target Matrix	Signature peptide	*pI*[1]	GRAVY[2]
CD3	Target/Biomarker, mouse	Mouse plasma	**ETS**NPLQVYYR	6.10	-1.15
IL-33	Biomarker, human	Mouse liver	**TDP**GVFIGVK	5.50	0.52
T84.66	Murine IgG	Mouse plasma, liver, tumor, muscle	**ASN**LESGIPVR	6.05	0.07
Carcinoembryonic antigen (CEA)	Target/Biomarker, human	Mouse tumor	**TLT**LISVTR	9.40	0.91
Anti-mCD3 antibody	Murine IgG	Mouse plasma	**LEI**LSQPK	6.00	-0.15
AB095	Humanized IgG	Mouse plasma	**GPS**VFPLAPSSK	8.75	0.09

[1]pI values were calculated by an online tool https://web.expasy.org/compute_pi/

[2]GRAVY values were acquired here http://www.gravy-calculator.de

Table S2. List of representative matrix peptides after tryptic digestion. All peptides were verified by tandem MS sequencing on an Orbitrap Fusion Lumos MS, and span a wide range of pI and GRAVY values.

a. Representative matrix peptides in liver sample

Protein Name	Peptide Sequence	pI	GRAVY[2]	Precursor
3-ketoacyl-CoA thiolase	LEDTLWAGLTDQHVK	4.54	-0.43	575.965
3-ketoacyl-CoA thiolase	TNVSGGAIALGHPLGGSGSR	9.44	0.08	904.474
3-ketoacyl-CoA thiolase	VGVPTETGALTLNR	5.97	0.13	714.396
4-trimethylaminobutyraldehyde dehydrogenase	GVKPITLELGGK	8.59	0.13	606.371
60 kDa heat shock protein	ISSVQSIVPALEIANAHR	6.75	0.49	953.031
60 kDa heat shock protein	TLNDELEIIEGMK	4	-0.31	752.882
60S acidic ribosomal protein P2	NIEDVIAQGVGK	4.37	0.04	621.838
Actin	DLYANTVLSGGTTMYPGIADR	4.21	-0.07	2215.072
Actin	EITALAPSTMK	6.1	0.24	581.313
Actin	GYSFTTTAER	6	-0.8	566.767
Actin	HQGVMVGMGQK	8.76	-0.28	391.195
Actin	SYELPDGQVITIGNER	4.14	-0.64	597.635
Actin	VAPEEHPVLLTEAPLNPK	4.75	-0.27	977.536
ATP synthase subunit alpha	ILGADTSVDLEETGR	3.92	-0.23	788.397
ATP synthase subunit alpha	TGAIVDVPVGEELLGR	4.14	0.5	812.949
ATP synthase subunit alpha	TSIAIDTIINQK	5.5	0.27	658.875
ATP synthase subunit alpha, mitochondrial	VVDALGNAIDGK	4.21	0.43	586.320
ATP synthase subunit b	HVVKSISVQQEK	8.6	-0.5	691.394
ATP synthase subunit beta	LVLEVAQHLGESTVR	5.4	0.38	825.962
ATP synthase subunit beta	SLQDIIAILGMDELSEEDK	3.71	-0.09	1068.025
ATP synthase subunit beta, mitochondrial	AIAELGIYPAVDPLDSTSR	4.03	0.21	663.349
ATP synthase subunit beta, mitochondrial	FTQAGSEVSALLGR	6	0.26	718.381
D-beta-hydroxybutyrate dehydrogenase	YEMHPLGVK	6.75	-0.44	1073.545
Electron transfer flavoprotein subunit alpha	LLYDLADQLHAAVGASR	5.21	0.35	906.984
Elongation factor 1-alpha 1	STTTGHLIYK	8.33	-0.34	560.803
Elongation factor 1-alpha 1	THINIVVIGHVDSGK	6.61	0.43	794.944
Elongation factor 1-alpha 1	VETGVLKPGMVVTFAPVNVTTEVK	6.11	0.61	1266.193
Elongation factor 1-alpha 1	YYVTIIDAPGHR	6.74	-0.13	702.867
Elongation factor 2	ARPFPDGLAEDIDK	4.23	-0.81	772.391
Enoyl-CoA hydratase, mitochondrial	AQFGQPEILLGTIPGAGGTQR	6.05	-0.1	1056.066
Glutamate dehydrogenase 1	IIAEGANGPTTPEADK	4.14	-0.56	792.399
Glyceraldehyde-3-phosphate dehydrogenase	GAAQNIIPASTGAAK	8.75	0.21	685.375
Glyceraldehyde-3-phosphate dehydrogenase	VIHDNFGIVEGLMTTVHAITATQK	5.99	0.43	1306.181
Glyceraldehyde-3-phosphate dehydrogenase	VIISAPSADAPMFVMGVNHEK	5.32	0.51	1123.053
Glyceraldehyde-3-phosphate dehydrogenase	WGEAGAEYVVESTGVFTTMEK	4.09	-0.16	1154.028
Malate dehydrogenase, mitochondrial	VDFPQDQLATLTGR	4.21	-0.39	780.904
Peroxiredoxin-5	THLPGFVEQAGALK	6.41	0.07	489.937
Protein disulfide-isomerase	LITLEEEMTK	4.25	-0.18	603.818
Protein disulfide-isomerase	THILLFLPK	8.44	1.03	361.230
Thiosulfate sulfurtransferase	TYEQVLENLQSK	4.53	-1.03	726.372

b. representative matrix peptides in plasma

Protein Name	Peptide Sequence	pI	GRAVY[2]	Precursor
Alpha-1-antitrypsin 1-3	DQSPASHEIATNLGDFAISLYR	4.54	-0.31	1203.096
Alpha-1-antitrypsin 1-3	FDHPFLFIIFEEHTQSPLFVGK	5.27	0.25	662.848
Alpha-1-antitrypsin 1-3	KPFDPENTEEAEFHVDESTTVK	4.25	-1.33	638.052
Alpha-1-antitrypsin 1-3	NHYQAEVFSVNFAESEEAK	4.48	-0.8	733.676
Alpha-1-antitrypsin 1-3	SFQHLLQTLNRPDSELQLSTGNGLFVNNDLK	5.36	-0.53	875.461
Apolipoprotein B-100	GLVHPLSTLISSSQTCQYTLDPK	6.73	-0.06	849.109
Apolipoprotein B-100	LDHTHSLNIAGLSLDFFSK	5.98	0.15	705.705
Apolipoprotein B-100	NNALHFLTTSYNEAK	6.75	-0.71	861.931
Complement C3	IILQGSPVVQMAEDAVDGER	3.92	0.11	709.701
Complement C3	SGIPIVTSPYQIHFTK	8.33	0.13	596.663
Complement C4-B	ASAGLLGAHAAAITAYALTLTK	8.64	1	695.731
Complement C4-B	STQDTVVTLDALSAYWIASHTTEEK	4.31	-0.27	922.794
Complement factor H	KPCGHPGDTPFGSFR	8.23	-0.97	553.931
Fibronectin	DTLTSRPAQGVITTLENVSPPR	6.07	-0.47	784.759
Fibronectin	GLTPGVIYEGQLISIQQYGHR	6.75	-0.18	777.086
Fibronectin	HALQSASAGSGSFTDVR	6.74	-0.19	564.280
Fibronectin	LRPRPYLPNVDEEVQIGHVPR	6.76	-0.84	497.678
Fibronectin	QDGHLWCSTTSNYEQDQK	4.54	-1.72	1098.971
Fibronectin	RPGAAEPSPDGTTGHTYNQYTQR	6.75	-1.67	835.395
Fibronectin	SDNVPPPTDLQFVELTDVK	3.84	-0.47	705.365
Fibronectin	VTWAPPPSIELTNLLVR	5.97	0.39	636.034
Fibronectin	VVTPLSPPTNLHLEANPDTGVLTVSWER	4.65	-0.13	1014.874
Hemopexin	ELGSPPGISLETIDAAFSCPGSSR	4.14	-0.01	816.733
Hemopexin	GATYAFTGSHYWR	8.6	-0.6	506.240
Hemopexin	LFQEEFPGIPYPPDAAVECHR	4.4	-0.43	824.732
Murinoglobulin-2	AQVLGYTSATTTDQHGLAK	6.79	-0.32	654.673
Murinoglobulin-2	ESVVFVQTDKPVYKPGQSVK	8.53	-0.46	559.559
Murinoglobulin-2	QQNSNGGFSSTQDTVVALDALSK	4.21	-0.5	1184.080
Serotransferrin	LYLGHSYVTAIR	9.18	0.49	696.885
Serotransferrin	AVLTSQETLFGGSDCTGNFCLFK	4.37	0.35	851.405
Serotransferrin	DFASCHLAQAPNHVVVSR	6.91	0.14	670.003
Serotransferrin	KPVDQYEDCYLAR	4.56	-1.1	828.888
Serotransferrin	LCQLCPGCGCSSTQPFFGYVGAFK	7.88	0.53	914.410
Serum albumin	ALVAAVR	10.55	1.87	350.229
Serum albumin	APQVSTPTLVEAAR	6.34	0.55	720.400
Serum albumin	AWAVAR	10.55	0.7	337.193
Serum albumin	DVFLGTFLYEYSR	4.19	1.11	537.271
Serum albumin	LVQEVTDFAK	4.19	0.17	575.310
Serum albumin	AHCLSEVEHDTMPADLPAIAADFVEDQEVCK	4.07	-0.1	1166.533
Serum albumin	RPCFSALTVDETYVPK	60.6	-1.08	628.321

[1]pI values were calculated by an online tool https://web.expasy.org/compute_pi/

[2]GRAVY values were acquired here http://www.gravy-calculator.de

Table S3. Simulated retention-related characteristics of tryptic peptide on CX-RP sorbent under different pH

pH	Predicted net charge*	Optimal for	Retention on CX-RP sorbent**	
			Cationic interaction	Hydrophobic interaction
pH=pI-2	~99% positively charged	SCX-wash	+++	+
pH=pI-1	~90% positively charged	---	++	++
pH=pI	No net charge	RP wash	-	+++++
pH=pI+1	~90% negatively charged	---	-	++
pH=pI+2	~99% negatively charged	Selective Elution	-	+

*Calculation based on Henderson-Hasselbalch equation

** Not to scale

Table S4. The universal procedure for development of optimal enrichment conditions. A strong elution (80% MeOH, 0.1% NH3 strong elution) is performed for optimization of CX and RP wash steps, and a generic, weak wash (0.1% formic acid in 5% methanol) is used for optimization of selective elution.

Optimization of CX wash.	
i	Under the optimal pH=pI-2 of the peptide, use a high ionic strength (*e.g.*, 250 mM salt), adjust the organic composition with a step size of 5%; select the organic solvent fraction achieving 80% desorption of target SP;
ii	With the identified optimal organic solvent%, survey concentrations of salt at a step size of 25-50mM; select the optimal ionic strength that the recovery of target is at least 70%, and the highest S/N is achieved;
Optimization of RP wash	
	Under the optimal pH (pI of target) and high, constant ionic strength (*e.g.*, 250 mM NaCl), optimize the organic solvent fraction with a step size of 5%; select the optimal condition that the recovery of target is at least 70% and highest S/N;
Optimization of Selective Elution	
	Under the optimal pH for target peptide (pI+2), adjust the organic composition with a step size of 5% using the buffer containing LC-MS compatible buffer salt (*e.g.*, 50mM ammonium acetate), select the optimal organic solvent that the recovery of target is at least 80%, and the highest S/N is achieved

Table S5. Proper buffer systems for pH regulation of CX-RP enrichment*

Buffer system	Target pH range
Ammonium formate	2.8-4.8
Ammonium acetate	3.6-5.6
Potassium Phosphate	5.8-8.0
Sodium Citrate	3.0-6.2
Tris	7.2-9.0
Ammonium bicarbonate	9.2-10.6

*Buffer salt concentration can be higher for CX-/RP-wash, and only the LC-MS compatible buffer salts are used for selective elution.

Supplemental Figures

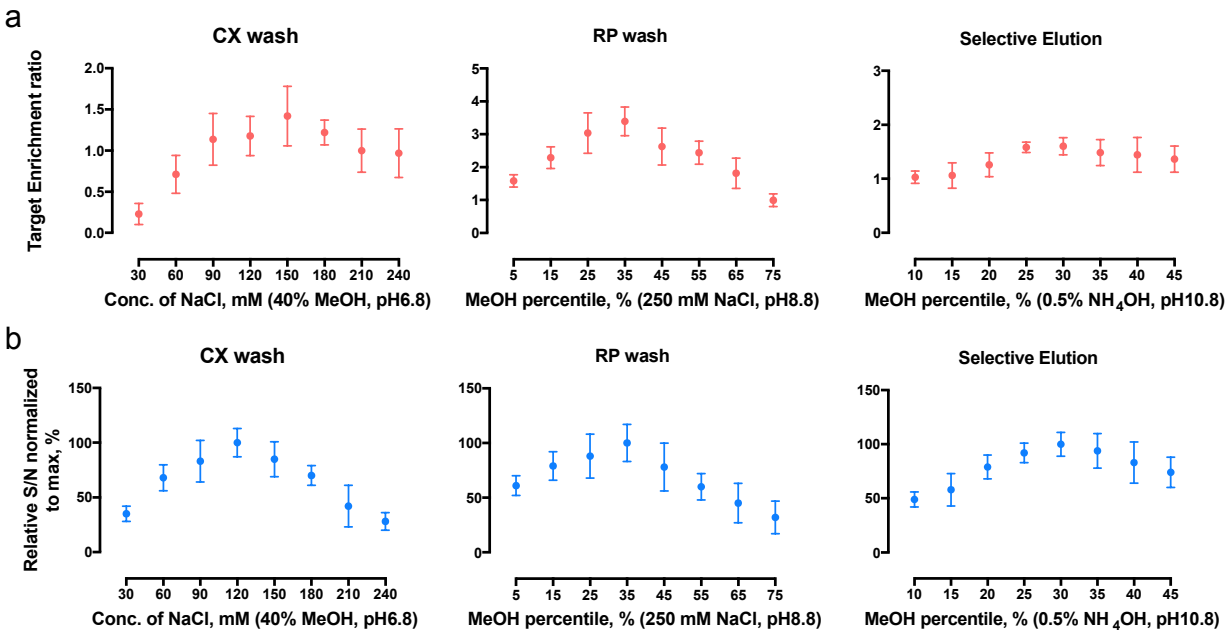

Figure S1. Target-enrichment-ratio (TER) and S/N for target SP shared very similar trends under varying conditions for washing and elution steps, exemplified by GPS peptide (n=3). To benchmark the efficiency of removing matrix peptides, we simultaneously monitored the desorption/recovery of ~40 representative matrix peptides from tissue or plasma along with the target SP during method development. These peptides are mostly from high-abundance matrix proteins and span a wide range of polarity and pI (**Table S2**), and therefore are representative of the matrix peptides to be eliminated.

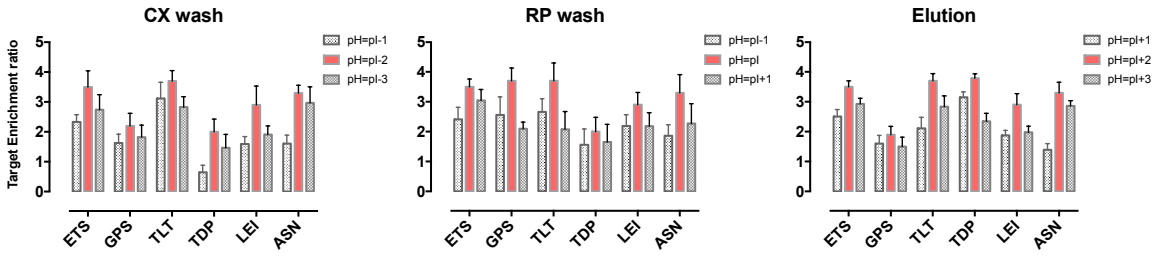

Figure S2. Target-enrichment-ratios of different pH for individual washing and elution steps (n=3).

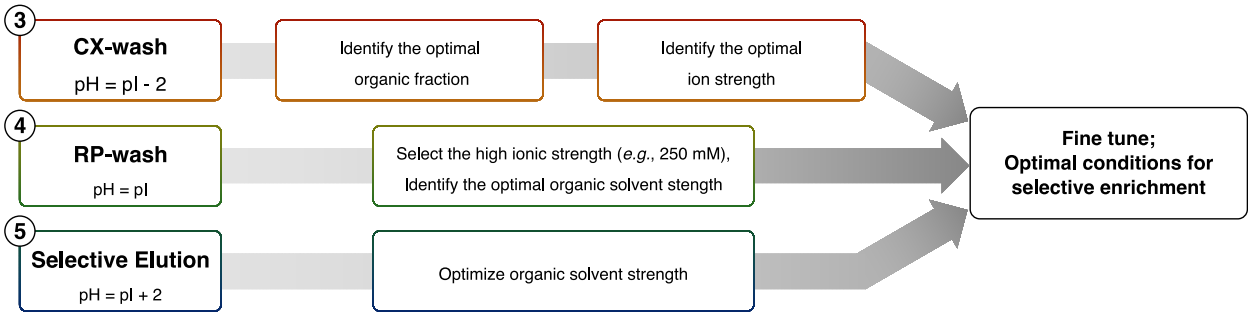

Figure S3. Optimization workflow for specific conditions for CX-/RP- washes and elution (step 3-5 in **Figure 2**).

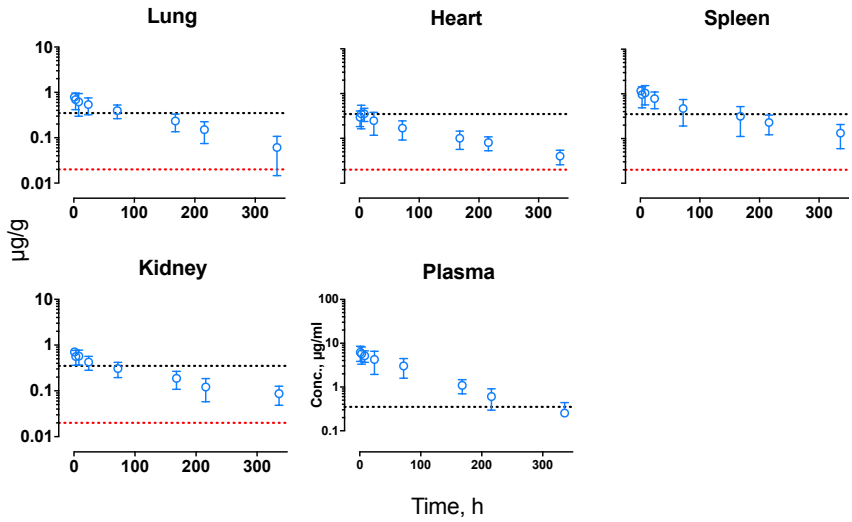

Figure S4. Additional PK profile of T84.66 in plasma and tissues after single I.V. injection of 0.5 mg/kg, n=3.

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