Supporting Information

Rapid High-pH Reverse Phase StageTip for Sensitive Small-Scale Membrane Proteomic Profiling

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I. Protocols for StageTip Fractionation

A. SAX StageTip Fractionation

Materials and Reagents:

1. SAX Empore disk
2. 16-gauge blunt-tipped needle
3. 200-µL Gilson pipet tips
4. 100% methanol
5. Solutions for conditioning and elution
   - Buffer 1 - 0.1% NH_4OH, 15% ACN
   - Buffer 2 - 0.1% NH_4OH, 80% ACN
   - Buffer 3 - 0.1% NH_4OH, 20mM $NH_4C_2H_3O_2$, 15% ACN
   - Buffer 4 - 0.1% NH_4OH, 50mM $NH_4C_2H_3O_2$, 15% ACN
   - Buffer 5 - 0.1% NH_4OH, 100mM $NH_4C_2H_3O_2$, 15% ACN
   - Buffer 6 - 0.1% NH_4OH, 500mM $NH_4C_2H_3O_2$, 15% ACN

Conditioning and equilibration were done through sequential passing and centrifugation at 1000 × *g* for 1 min of the following reagents:

1. 50 µL of 100% methanol
2. 50 µL of Buffer 2
3. 50 µL of Buffer 1
4. 50 µL of Buffer 6 (soak for 10 min before centrifugation)
5. 50 µL of Buffer 1

After conditioning and equilibration, 10 µg of membrane peptide digests from HeLa cell line, desalted using SDB StageTip and dissolved in 50 µL of Buffer 1, were bound to the SAX membrane through centrifugation at 1000 × *g* for 1 min with the eluate collected as flowtrough (FT). The bound peptides were washed with 50 µL of Buffer 1 by centrifugation at 1000 × *g* for 1 min, collecting the eluate into the same tube as FT (FT/wash = combined flowtrough and wash). Peptide separation is achieved through sequential elution with 50 µL of Buffer 3 to Buffer 6 to obtain F1 (Buffer 3), F2 (Buffer 4), F3 (Buffer 5), and F4 (Buffer 6) by centrifugation at 1000 × *g* for 1 min in each fraction.

B. SCX StageTip Fractionation

Materials and Reagents:

1. SCX Empore disk

2. 16-gauge blunt-tipped needle
3. 200-µL Gilson pipet tips
4. 100% methanol
5. Solutions for conditioning and elution
    - Buffer A - 0.1% TFA, 5% ACN
    - Buffer B - 0.1% TFA, 80% ACN
    - Buffer 0 - 0.1% TFA, 15% ACN
    - Buffer 20 - 0.1% TFA, 20mM $NH_4C_2H_3O_2$, 15% ACN
    - Buffer 40 - 0.1% TFA, 40mM $NH_4C_2H_3O_2$, 15% ACN
    - Buffer 80 - 0.1% TFA, 80mM $NH_4C_2H_3O_2$, 15% ACN
    - Buffer 200 - 0.1% TFA, 200mM $NH_4C_2H_3O_2$, 15% ACN
    - Buffer 500 - 0.1% TFA, 500mM $NH_4C_2H_3O_2$, 15% ACN

Conditioning and equilibration were done through sequential passing and centrifugation at 1000 × *g* for 1 min of the following reagents:

1. 50 µL of 100% methanol
2. 50 µL Buffer B
3. 50 µL Buffer A
4. 50 µL Buffer 500 (soak for 10 min before centrifugation)
5. 50µL Buffer 0

The same procedure as with the SAX StageTip was done for binding 10 µg of desalted membrane peptide digests from HeLa cell line dissolved in Buffer 0 to the SCX StageTip. Wash the peptides by passing the tip with Buffer 0 through centrifugation at 1000 × *g* for 1 min, collecting the eluate into the FT fraction (FT/wash). Peptide separation is through sequential elution with 50 µL of Buffer 20 to Buffer 500 to obtain F1 (Buffer 20), F2 (Buffer 40), F3 (Buffer 80), F4 (Buffer 200), and F5 (Buffer 500) by centrifugation at 1000 × *g* for 1 min in each fraction.

C. Hp-RP StageTip Fractionation Using Copolymer-based SDB-XC Separation Material

Materials and Reagents:

1. SDB-XC Empore disk
2. 16-gauge blunt-tipped needle
3. 200-µL Gilson pipet tips
4. 100% methanol

5. Solutions for conditioning and elution
    - Buffer A – 200 mM NH_4HCO_2 (pH 10)
    - Buffer B - 100% ACN
    - Buffer F1 – 11.1% buffer B in Buffer A
    - Buffer F2 – 14.5% buffer B in Buffer A
    - Buffer F3 - 17.4% buffer B in Buffer A
    - Buffer F4 - 20.8% buffer B in Buffer A
    - Buffer F5 - 45.0% buffer B in Buffer A
    - Buffer F6 - 65.0% buffer B in Buffer A
    - Buffer F7 - 80.0% buffer B in Buffer A

Conditioning and equilibration were done through sequential passing and centrifugation at 1000 × *g* for 1 min of the following reagents:

1. 50 µL of 100% methanol
2. 50 µL Buffer F7
3. 50 µL Buffer F4
4. 50 µL Buffer A (soak for 10 min before centrifugation)

The same procedure as with the SAX and SCX StageTips was done for binding 10 µg of desalted membrane peptide digests from HeLa cell line dissolved in 50 µL of Buffer A to the Hp-RP StageTip. The peptides were washed with 50 µL of Buffer A by centrifugation at 1000 × *g* for 1 min. The eluate was collected into the FT tube to obtain the FT/wash fraction. Peptide separation is through sequential elution with 50 µL of Buffer F1 to Buffer F7 to obtain F1 (Buffer F1), F2 (Buffer F2), F3 (Buffer F3), F4 (Buffer F4), F5 (Buffer F5), and F6 (Buffer F6 and Buffer F7 combined) by centrifugation at 1000 × *g* for 1 min in each fraction.

D. Hp-RP StageTip Fractionation Using Particle-based C18-AQ Separation Material

Materials and Reagents:

1. 1.25 mg of C18-AQ (5 µm)
2. C8 Empore disk
3. 18-gauge blunt-tipped needle
4. 200-µL Gilson pipet tips
5. 100% methanol
6. Solutions for conditioning and elution

- Buffer A – 200 mM NH_4HCO_2 (pH 10)
- Buffer B - 100% ACN
- Buffer F1 – 11.1% buffer B in Buffer A
- Buffer F2 – 14.5% buffer B in Buffer A
- Buffer F3 - 17.4% buffer B in Buffer A
- Buffer F4 - 20.8% buffer B in Buffer A
- Buffer F5 - 45.0% buffer B in Buffer A
- Buffer F6 - 65.0% buffer B in Buffer A
- Buffer F7 - 80.0% buffer B in Buffer A

The same conditioning and equilibration procedure were done as with the Hp-RP StageTip using SDB material with centrifugation at 1500 × *g* for 2 min in each step. The procedure for peptide binding, washing, and fractionation of the 10 µg membrane peptide digests from HeLa cell line and 11 lung cancer cell lines were the same as described in the protocol of Hp-RP StageTip using SDB-XC material.

II. Tissue Culture and Lysis

Lung tissue samples from male Sprague-Dawley (SD) rats were obtained from Dr. Yuh-Chang Sun, National Tsing Hua University. Frozen tissues were thawed rapidly at 37 °C, cut into small pieces, weighed and then washed by 0.9% NaCl to remove blood. The pre-cleaned tissues were homogenized in STM buffer solution (5mL/g tissue, 0.25 M sucrose, 10 mM Tris-HCl, and 1 mM $MgCl_2$) with protease inhibitor mixture (100:1, sample/protease inhibitor, v/v) using mechanical homogenizer (Precellys®24, Bertin Technologies) in 2.0 mL standard tubes (STURDY TUBE®) containing ceramic zirconium oxide beads (5 beads of 2.8 mm diameter and 10 beads of 1.4 mm diameter). The tissue samples were homogenized at 6500 rpm three times for 15 seconds pausing for 5 min in between each homogenization steps, and then tissue debris was removed by centrifugation (260 × g) for 5 min at 4 °C. The supernatant was centrifuged at 1500 × g for 10 min at 4 °C to pellet the nucleus, and then the obtained supernatant was centrifuged again at 13000 rpm for 1 h. at 4 °C to precipitate the crude membrane pellet. The pellet was washed in 1 mL of 0.1 M Na_2CO_3 overnight at 4 °C and re-collected by centrifugation at 13000 rpm for 1 h. at 4 °C.

III. Data Acquisition and LC Gradient for Synapt G1 HDMS and AB SCIEX 5600 TripleTOF System Analysis

For LC-MS/MS analysis through Synapt G1 HDMS, the fractionated peptides reconstituted in buffer A (0.1% FA in H_2O) were injected into a 2 cm × 180 µm capillary trap column and separated by 75 µm ×

25 cm nanoACQUITY 1.7 µm BEH C18 column using nanoACQUITY Ultra Performance LC™ (Waters Corp., Milford, MA). For the analysis using AB SCIEX TripleTOF 5600 System, peptide samples were injected in a 100 µm × 150 mm self-packed 3 µm C18-AQ column in a nanoACQUITY Ultra Performance LC™. The bound peptides were eluted with a gradient of 0-80% buffer B (0.1% FA in ACN) for 120 min, operated in ESI-positive V mode. Data acquisition for Synapt G1 HDMS was done by data-dependent acquisition mode to automatically switch between a full MS scan (400-1600 m/z, 0.6 s.) and six MS/MS scans (100-1990 m/z, 0.6 s. for each scan) on the six most intense ions present. Data from AB SCIEX 5600 TripleTOF System were obtained through the information-dependent acquisition mode by selecting 15 most intense precursor peaks and performing 15 MS/MS (100-1800 m/z, 200 ms. for each scan) for each full MS scan (300-1600 m/z, 200 ms.).

A. **LC Gradient for SAX and SCX Fractions in Synapt G1 HDMS** (Buffer A = 0.1% FA in H_2O, Buffer B = 0.1% FA in ACN)

All Fractions	
Time (min)	% Buffer B
1	5
38	15
55	20
65	25
75	30
90	35
95	40
103	45
108.5	80
110	80
110.5	1

B. **LC Gradient for Hp-RP Fractions in Synapt G1 HDMS** (Buffer A = 0.1% FA in H_2O, Buffer B = 0.1% FA in ACN)

FT/Wash and F1		F2		F3		F4		F5 and F6	
Time (min)	% Buffer B	Time (min)	% Buffer B	Time (min)	% Buffer B	Time (min)	% Buffer B	Time (min)	% Buffer B
1	3	1	5	1	5	1	5	1	10
15	8	15	8	20	10	10	10	20	20
35	10	30	10	40	15	30	15	50	25
55	15	55	15	60	20	45	20	75	30
75	20	75	20	80	25	80	30	85	35
90	25	90	25	95	30	105	40	95	40
100	35	100	30	105	40	108.5	80	105	45
105.5	45	105	40	108.5	80	110	80	108.5	80
108.5	80	108.5	80	110	80	110.5	1	110	80
110	80	110	80	110.5	1			110.5	1
110.5	1	110.5	1						

C. LC Gradient for Hp-RP fractions in AB SCIEX TripleTOF 5600 System (Buffer A = 0.1% FA in H_2O, Buffer B = 0.1% FA in ACN)

FT/Wash and F1		F2		F3		F4		F5 and F6	
Time (min)	% Buffer B	Time (min)	% Buffer B	Time (min)	% Buffer B	Time (min)	% Buffer B	Time (min)	% Buffer B
0	1	0	1	0	1	1	1	0	1
1	3	1	5	1	5	1	8	1	10
15	8	20	10	15	10	25	15	20	20
35	10	40	15	35	15	40	20	50	25
55	15	60	20	55	20	65	25	75	30
75	20	80	25	75	25	80	30	85	35
90	25	95	30	90	30	105	40	95	40
100	35	105	40	105	40	108.5	80	105	45
105.5	45	108.5	80	108.5	80	110	80	108.5	80
108.5	80	110	80	110	80	110.5	1	110	80
110	80	110.5	1	110.5	1			110.5	1
110.5	1								

IV. LC Gradient for Analysis in LTQ Orbitrap Fusion (Buffer A = 0.1% FA in H_2O, Buffer B = 0.1% FA in ACN)

A. LC Gradient for Hp-RP fractions

FT/Wash and F1		F2		F3		F4		F5 and F6			
Time (min)	% Buffer B	Time (min)	% Buffer B	Time (min)	% Buffer B	Time (min)	% Buffer B	Time (min)	% Buffer B	Time (min)	% Buffer B
1	1	1	1	1	1	1	1	1	1	1	1
2	2	2	2	2	2	2	2	2	2	2	2
110	15	105	20	105	20	115	30	5	10	105	20
120	25	115	30	115	30	125	40	25	20	115	30
130	40	125	40	125	40	132	80	60	25	125	40
132	80	132	80	132	80	137	80	95	30	132	80
137	80	137	80	137	80	138	1	105	35	137	80
138	1	138	1	138	1	150	1	115	40	138	1
150	1	150	1	150	1			116	80	150	1
								121	80		
								122	1		
								150	1		

B. LC Gradient for SAX and SCX fractions

All Fractions	
Time (mins.)	% Buffer B
1	1
2	2
112	25
122	35
123	90
129	90
130	1
150	1

V. Database Search and Confidence Filtering for Synapt G1 HDMS and AB SCIEX TripleTOF 5600 Data

The acquired MS/MS spectra were searched against Swiss-Prot human database (version 57.8, 20,102 entries) using Mascot (Matrix Science; version 2.3.02). Database search of the spectra obtained from Synapt G1 HDMS were performed with both a parent ion and fragment ion mass tolerance of 0.1 Da. For spectra obtained from AB SCIEX TripleTOF 5600 System, a fragment ion mass tolerance of 0.1 Da and a parent ion tolerance of 20 ppm were used. Two missed cleavages were allowed for trypsin digestion. Deamidation (Asn and Gln), oxidation (Met) and methylthio (Cys) were selected as variable modifications. Peptide level FDR < 1% ($p < 0.05$) using Percolator scoring was employed for the membrane proteomic profiling of the 11 lung cancer cell lines.

VI. Andromeda Searching and Label-Free Quantitation Parameters by MaxQuant

Identifications were obtained through the Andromeda search engine using the same variable modifications as specified above. Mass tolerance was set to 0.07 Da and 40 ppm for the peptide precursor and fragment ion, respectively. A maximum of two missed cleavages, and protein and peptide FDR of 1% were allowed for peptide and protein identifications. Quantification was done through label-free quantification (LFQ). LFQ values were transformed to Log_2, and then missing values were replaced with intensities in the lower region of the normal distribution (width of 0.3 and down shift of 1.8).

VII. Membrane Protein and TMH Annotation Using the HuMemProtDB

Membrane protein annotation is based on four categories, and proteins that matched with one of the following categories were considered as membrane protein:

1. Membrane annotations based on Gene Ontology (GO), Human Protein Reference Database (HPRD), and the Human Protein Atlas (HPA)

2. Annotations using subcellular localization predictors: PredKnow, PROlocalizer, CELLO, and WoLF PSORT
3. TMH Predictors: TMHMM, SWMTOP, Phobius, and Scampi
4. Glycosylphosphatidylinositol (GPI)-anchored proteins predictors, PTM annotations, and protein to protein interactions: GPI-SOM, PredGPI, FragAnchor, MeTaDor and Uniprot, and Biogrid PPI

A protein annotated as membrane protein is considered to have TMH domain if found in Uniprot and in at least one of the four TMH predictors: TMHMM, SWMTOP, Phobius, and Scampi.

Table S1. Medical Information on the 11 NSCLC Cell Lines (ADC: adenocarcinoma, SCC: squamous carcinoma).

Cell Line	Gender	Histology	EGFR Status	Response to Gefitinib
CL141	M	ADC	Wild Type	Resistant
CL152	M	SCC	Wild Type	Resistant
A549	M	ADC	Wild Type	Resistant
CL83	M	ADC	Wild Type	Resistant
CL100	F	SCC	Exon 19 Deletion	Partial Resistance
CL25	M	ADC	Exon 19 deletion	Partial Resistance
PC9	F	ADC	Exon 19 deletion	Sensitive
PC9/gef	F	ADC	Exon 19 deletion	Resistant
H1975	F	ADC	L858R/T790M	Resistant
CL97	M	ADC	G719A/T790M	Resistant
H3255	F	ADC	L858R	Sensitive

Table S2. Number of Proteins, Peptides, Membrane Proteins, Integral Membrane Proteins, and Integral Plasma Membrane Proteins Identified in Each Replicates of SAX, SCX and Hp-RP StageTip (C18) Methods using 10 µg of Membrane Protein Digests from HeLa Cell Line.

Category	Hp-RP Replicates			SCX Replicates			SAX Replicates		
	1	2	3	1	2	3	1	2	3
Proteins	4397	4460	4515	3229	3060	3317	3581	3573	3911
Peptides	19591	20019	20381	10972	10031	11517	11615	11486	13228
Membrane proteins	2845	2900	2906	2208	2100	2263	2403	2386	2577
Integral Membrane Proteins	1283	1292	1288	985	952	1023	1068	1080	1140
Integral Plasma Membrane Proteins	620	632	623	504	496	512	530	554	491

Table S3. Membrane Proteomic Analysis of Lung Tissue Samples from SD Rat.

Category	**Identifications**
Total Proteins	3411
Total Peptides	10310
Membrane Proteins	2317
Integral Membrane Proteins	1099
Peptides Located at TMH Regions	374

Table S4. List of Biological Pathways Associated with The Common Up-regulated and Down-regulated Membrane Proteins Found in CL100, CL25 and PC9/gef relative to PC9.

Up-regulated Membrane Proteins						
Category	**Terms**	**Count**	**P-value**	**Enrichment fold**	**Bonferroni**	**Benjamini**
KEGG_PATHWAY	Focal adhesion	8	8.33E-04	4.9	4.56E-02	1.16E-02
KEGG_PATHWAY	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	6	2.86E-04	9.79	1.59E-02	7.97E-03
KEGG_PATHWAY	Dilated cardiomyopathy	6	6.94E-04	8.09	3.81E-02	1.29E-02
KEGG_PATHWAY	Regulation of actin cytoskeleton	6	2.54E-02	3.46	7.64E-01	1.48E-01
KEGG_PATHWAY	Hypertrophic cardiomyopathy	5	4.20E-03	7.30	2.10E-01	4.61E-02
KEGG_PATHWAY	ECM-receptor interaction	4	2.77E-02	5.91	7.93E-01	1.46E-01
KEGG_PATHWAY	Pathogenic Escherichia coli infection	12	7.34E-02	6.53	9.86E-01	2.63E-01
Down-regulated Membrane Proteins						
KEGG_PATHWAY	Oxidative phosphorylation	11	4.42E-07	8.27	2.74E-05	2.74E-05
KEGG_PATHWAY	Alzheimer's disease	8	0.001078	4.80	0.06469531	0.032888482
KEGG_PATHWAY	Parkinson's disease	7	0.001609779	5.35	0.09506005	0.032747369
KEGG_PATHWAY	Huntington's disease	7	0.008677622	3.80	0.41746177	0.126362824

Table S5. Log_2 of LFQ Ratios for Selected Common Up-regulated and Down-regulated Proteins in PC9/gef, CL25 and CL100 Associated to Different Types of Cancer.

Gene Names	Significant Log_2 LFQRatio						Reference Literature
	PC9/gef /PC9	p-value	CL25 /PC9	p-value	CL100 /PC9	p-value	
MCAM	6.4	2.8E-4	4.9	9.6E-05	4.3	3.6E-3	Lung cancer[1-3], Breast cancer[4]
ALCAM	4.6	6.6E-2	5.7	2.9E-2	4.9	4.1E-2	Prostate cancer[5], Colorectal cancer[6], Breast cancer[7], Gastric cancer[8], Thyroid cancer[9], Ovarian cancer[10]
ABCC3	7.0	2.6E-3	5.1	1.0E-2	4.5	1.2E-2	Lung cancer,[11] Breast cancer[12]
ITGB4	6.2	8.3E-3	6.6	6.5E-3	6.3	7.3E-3	Breast cancer,[13] Lung cancer,[14] Prostate cancer[15]
PTGES	2.8	1.1E-1	3.3	7.9E-2	3.4	7.9E-2	Lung cancer,[16, 17] Breast cancer[18]
FLNA	3.6	2.6E-3	2.7	4.5E-3	3.7	2.1E-3	Lung cancer,[19] Hepatocellular carcinoma,[20] Breast cancer[21]
LSR	3.8	1.4E-2	3.4	3.0E-2	2.7	2.3E-2	Breast cancer[22]
UTRN	2.4	1.3E-1	2.7	5.9E-3	1.8	3.9E-3	Ovarian and Breast cancer[23]
SDHD	-3.2	3.4E-2	-3.5	2.9E-2	-3.9	2.1E-2	Tumorigenesis[24]
RAB11FIP1	-1.1	3.3E-2	-1.9	4.4E-2	-1.8	2.5E-2	Bladder cancer[25]
ACO2	-1.5	4.7E-2	-1.0	1.0E-1	-1.7	4.6E-2	Gastric cancer[26]
MFN2	-2.2	6.7E-2	-6.1	1.0E-3	-4.1	1.2E-2	Gastric cancer[27]
NOTCH2	-2.3	3.6E-2	-2.5	2.3E-2	-1.9	2.0E-2	Ovarian cancer,[28] Lung cancer,[29] Astrocytic Gliomas,[30] Gastric cancer,[31] Bladder cancer[32]

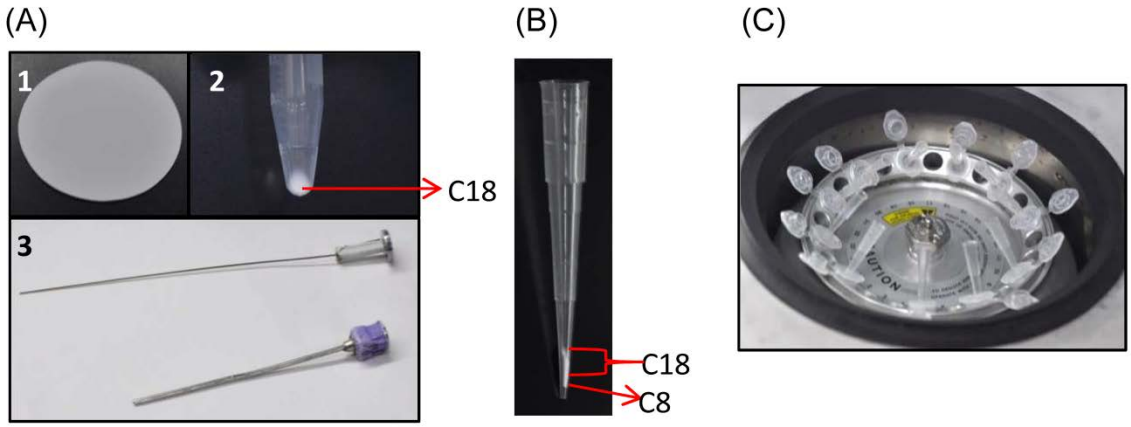

Figure S1. (A) Materials used for Hp-RP (C18) StageTip preparation: 1. C8 membrane used as frit, 2. C18 beads for fractionation, and 3. 18-gauge needle and thin tubing for inserting the C8 frit into the 200-μL tip. (B) Packed C18 StageTip. (C) Hp-RP StageTip fractionation through multiplexed processing.

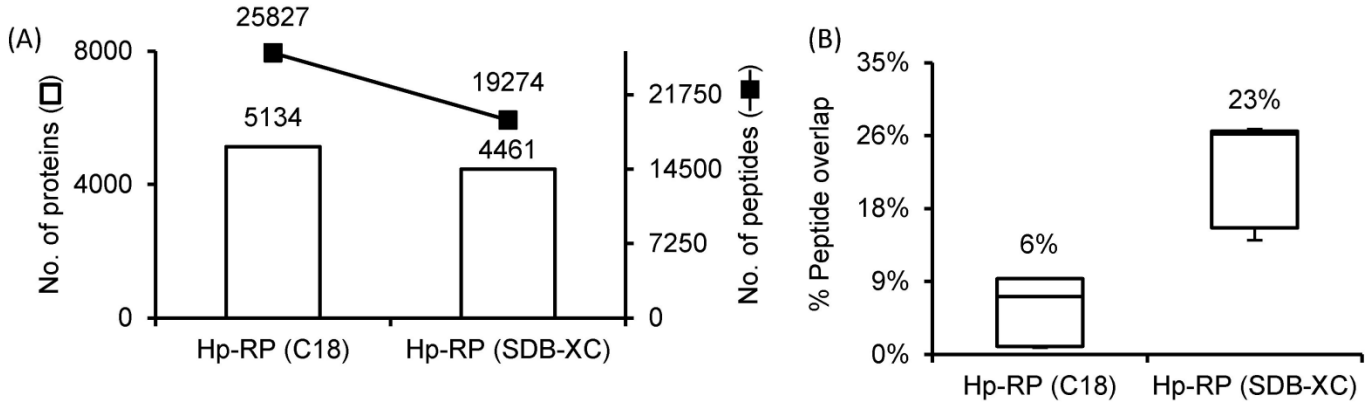

Figure S2. (A) Comparison of peptide and protein identification results in Hp-RP StageTip fractionation by using either particle-based C18 or copolymer-based SDB-XC. (B) Comparison of separation efficiency using copolymer-based SDB-XC and particle-based C18 for Hp-RP StageTip fractionation showing superior separation efficiency with the particle-based C18

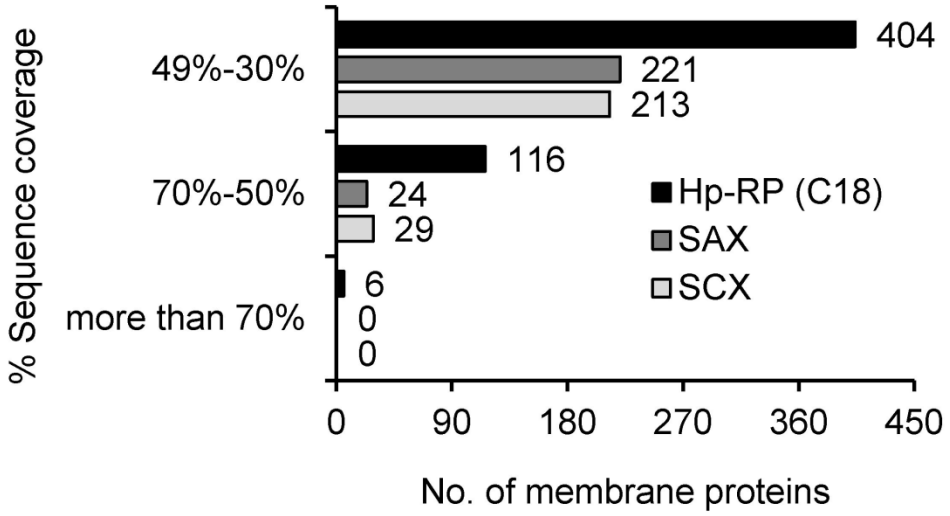

Figure S3. Comparison of sequence coverage (%) of the membrane proteins identified with Hp-RP (C18), SAX, and SCX fractionation methods.

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