Electronic Supplementary Material (ESI) for Analyst.


# A VERSATILE REVERSED PHASE-STRONG CATION EXCHANGE-REVERSED PHASE (RP-SCX-RP) MULTIDIMENSIONAL LIQUID CHROMATOGRAPHY PLATFORM FOR QUALITATIVE AND QUANTITATIVE SHOTGUN PROTEOMICS

**Supplementary information 1**

*Isolation of chloroplasts and the protein extraction procedure*

In the extraction of chloroplast proteins from plant tissues, ground tissue under liquid nitrogen was vortexed with 10% trichloroacetic acid in acetone. The mixture was centrifuged at 16000 × *g* for 5 minutes at 4°C. The pellet from previous step was then vortexed with 0.1 M $NH_4OAc$ in 80% methanol and centrifuged at 16000 × *g* for 5 min at 4°C. The supernatant was removed and SDT (4% SDS, 50 mM DTT and 0.1 M Tris-Cl pH 8.0) was added to the pellet. The mixture was homogenized and heated at 95°C for 10 minutes. Debris was removed by centrifugation at 16000 × *g* for 5 minutes at 4°C twice and the supernatant containing the protein was collected. Proteins were purified by acetone precipitation and dissolve in urea buffer (6 M urea in 200 mM MOPS/ 4 mM $CaCl_2$ pH 8.0).

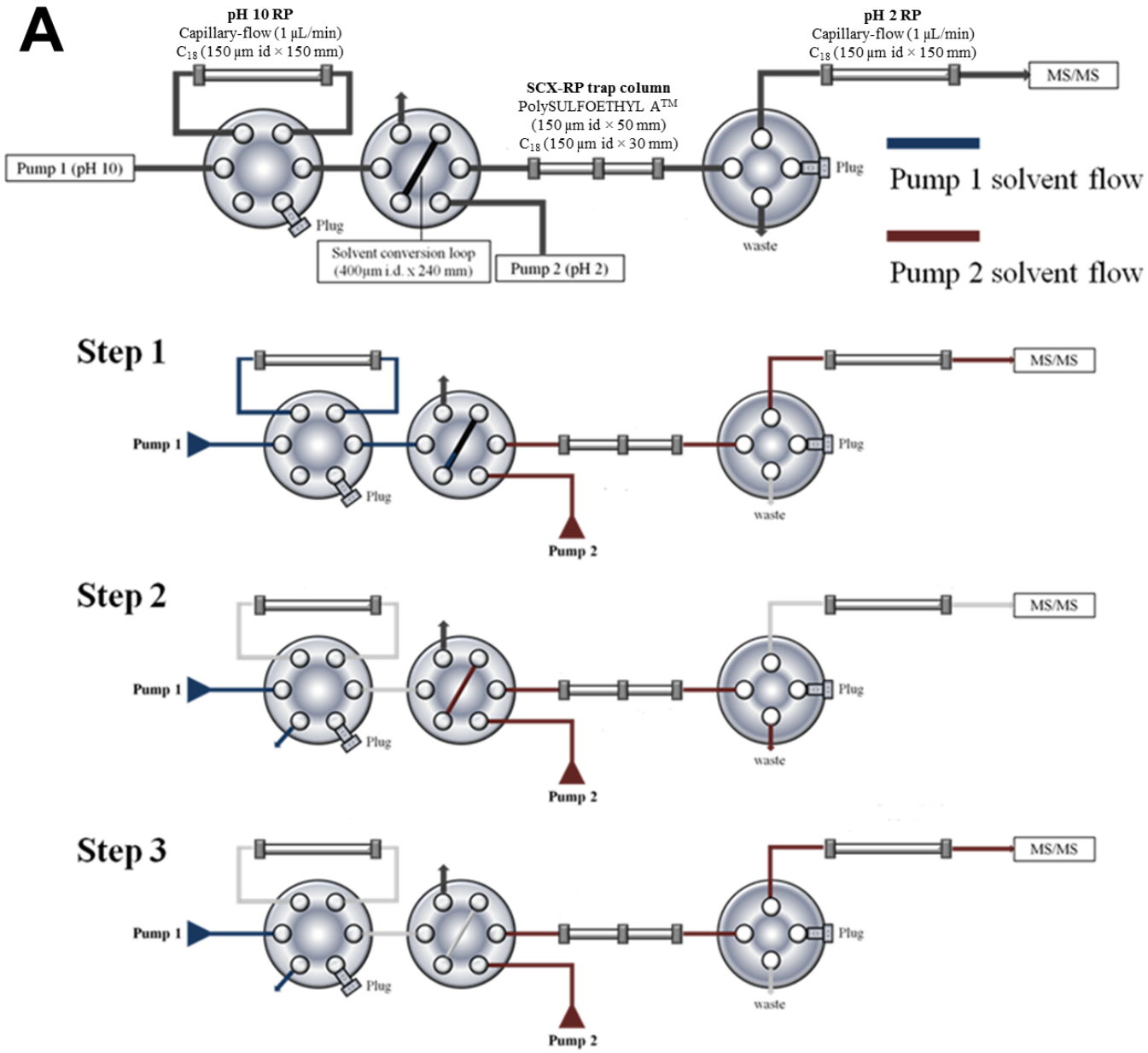
**Supplementary information 2**

*Online 2D RP–RP and 3D RP–SCX–RP analysis with the tailor-made platform*

The tailor-made platform consisting of 2 six-port and 1 four-port valves was assembled as shown as **Figure S-1A**. Peptides were dissolved in or diluted with buffer A and then loaded onto the first dimension RP column at 1 μL/min. The peptides were separated through pH 10 RP with a linear gradient from 0 to 45% buffer B using pH 10 RP buffers A and B at a flow rate of 1 μL/min. We employed eight fractionation steps for the pH 10 RP column in the first dimension. Every fraction of eluate from pH 10 RP was collected in 5 min intervals using a 30 μL mixing loop prefilled with buffer C. (**Figure S-1A**, **Step 1**) The stored fraction was then transferred to the SCX column with a stream of 100% buffer C at 3 μL/min for 25 min. (**Figure S-1A**, **Step 2**) The peptides on the SCX column were eluted sequentially with three plugs of salt solution of increasing strength (20 mM, 100 mM and 1000 mM) in the second dimension. Meanwhile, the LC flow for the pH 10 RP was bypassed. Finally, peptides were eluted onto the RP trap column through an injection of ammonium buffer with 100% buffer C at 3 μL/min by the autosampler; the system was further equilibrated for 20 min. The peptides were then separated in the pH 2 RP column using a linear gradient; the gradient was varied according to the organic content of the mobile phase that eluted from the pH 10 RP (see **Table S-1** and **Table S-2**). The gradient length of pH 2 RP (last dimension) for the 2D RP–RP and 3D RP–SCX–RP systems were 180 and 90 min, respectively; the total mass

spectrometric acquisition time per analysis using either the 2D RP–RP or 3D RP–SCX–RP system was approximately 36 h.

**Figure S-1** (A) Schematic representation and operation of the tailor-made platform. **Step 1**: pH 10 RP fractionation; **Step 2**: online solvent mixing followed by SCX trapping of fractionated peptides; **Step 3**: SCX peptide sub-fractionation and pH 2 RP gradient separation. (B) Proportional Venn diagrams obtained from overlapping the proteins and peptides identified in the analysis of yeast tryptic digests with the 2D RP–RP implemented on the tailor-made and the commercial LC integrated platforms.



**B**

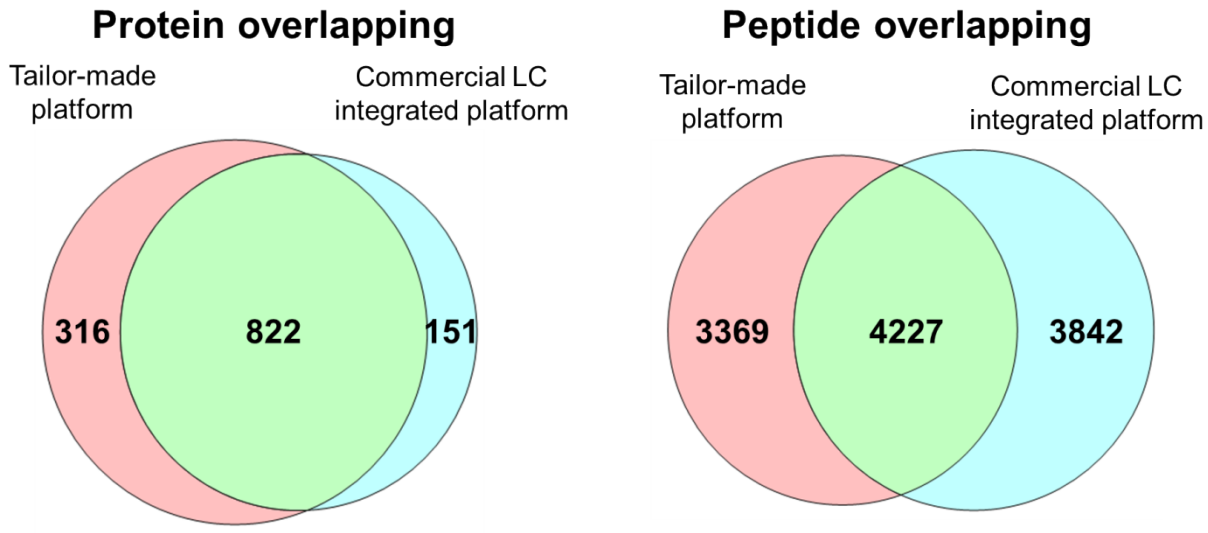

Protein overlapping
Tailor-made platform
Commercial LC integrated platform
316
822
151
Peptide overlapping
Tailor-made platform
Commercial LC integrated platform
3369
4227
3842

**Figure S-2.** The detailed stepwise procedure, valve positions and division of labor for the individual trap columns during the operation of (A) 2D RP–RP, (B) 3D RP–SCX–RP with 2 SCX sub-fractions and (C) 3D RP–SCX–RP with 3 SCX sub-fractions.

**A**

Repeat cycle until every fraction was analyzed

| | SCX-RP trap column 1 | SCX-RP trap column 2 | V1 | V2 |
|---|---|---|---|---|
| **Step 1** | Fractionate peptides from pH 10 RP | Idle | 1-2 | 1-2 |
| **Step 2** | Transfer peptides to SCX column | | 1-10 | |
| **Step 3** | Inject salt for peptide release | | 1-2 | |
| **Step 4** | Peptide separated and eluted out of pH 2 RP | Fractionate peptides from pH 10 RP | 1-2 | 1-10 |
| **Step 5** | | Transfer peptides to SCX column | 1-10 | |
| **Step 6** | | Inject salt for peptide release | 1-2 | |
| **Step 1** | Fractionate peptides from pH 10 RP | Peptide separated and eluted out of pH 2 RP | 1-2 | 1-2 |
| **Step 2** | Transfer peptides to SCX column | | 1-10 | |
| **Step 3** | Inject salt for peptide release | | 1-2 | |

**B**

Repeat cycle until every fraction was analyzed

| | SCX-RP trap column 1 | SCX-RP trap column 2 | V1 | V2 |
|---|---|---|---|---|
| **Step 1** | Fractionate peptides from pH 10 RP | Idle | 1-2 | 1-2 |
| **Step 2** | Transfer peptides to SCX column | | 1-10 | |
| **Step 3** | Inject salt for SCX fractionation | | 1-2 | |
| **Step 4** | Peptide separated and eluted out of pH 2 RP | Fractionate peptides from pH 10 RP | 1-2 | 1-10 |
| **Step 5** | | Transfer peptides to SCX column | 1-10 | |
| **Step 6** | | Inject salt for SCX fractionation | 1-2 | |
| **Step 3** | Inject salt for SCX fractionation | Peptide analyzed by pH 2 gradient pump | 1-2 | 1-2 |
| **Step 6** | Peptide analyzed by pH 2 gradient pump | Inject salt for SCX fractionation | 1-2 | 1-10 |
| **Step 1** | Fractionate peptides from pH 10 RP | Peptide separated and eluted out of pH 2 RP | 1-2 | 1-2 |
| **Step 2** | Transfer peptides to SCX column | | 1-10 | |
| **Step 3** | Inject salt for SCX fractionation | | 1-2 | |

**C**

Repeat cycle until every fraction was analyzed

| | SCX-RP trap column 1 | SCX-RP trap column 2 | V1 | V2 |
|---|---|---|---|---|
| **Step 1** | Fractionate peptides from pH 10 RP | Idle | 1-2 | 1-2 |
| **Step 2** | Transfer peptides to SCX column | | 1-10 | |
| **Step 3** | Inject salt for SCX fractionation | | 1-2 | |
| **Step 4** | Peptide separated and eluted out of pH 2 RP | Fractionate peptides from pH 10 RP | 1-2 | 1-10 |
| **Step 5** | | Transfer peptides to SCX column | 1-10 | |
| **Step 6** | | Inject salt for SCX fractionation | 1-2 | |
| **Step 3** | Inject salt for SCX fractionation | Peptide analyzed by pH 2 gradient pump | 1-2 | 1-2 |
| **Step 6** | Peptide analyzed by pH 2 gradient pump | Inject salt for SCX fractionation | 1-2 | 1-10 |
| **Step 3** | Inject salt for SCX fractionation | Peptide analyzed by pH 2 gradient pump | 1-2 | 1-2 |
| **Step 6** | Peptide analyzed by pH 2 gradient pump | Inject salt for SCX fractionation | 1-2 | 1-10 |
| **Step 1** | Fractionate peptides from pH 10 RP | Peptide separated and eluted out of pH 2 RP | 1-2 | 1-2 |
| **Step 2** | Transfer peptides to SCX column | | 1-10 | |
| **Step 3** | Inject salt for SCX fractionation | | 1-2 | |

**Figure S-3.** Schematic diagram showing the workflow of the first two dimensions of 3D RP–SCX–RP in the dual trap configuration with (A) 12 pH 10 RP fractions and 2 SCX sub-fractions or (B) 8 pH 10 RP fractions and 3 SCX sub-fractions.

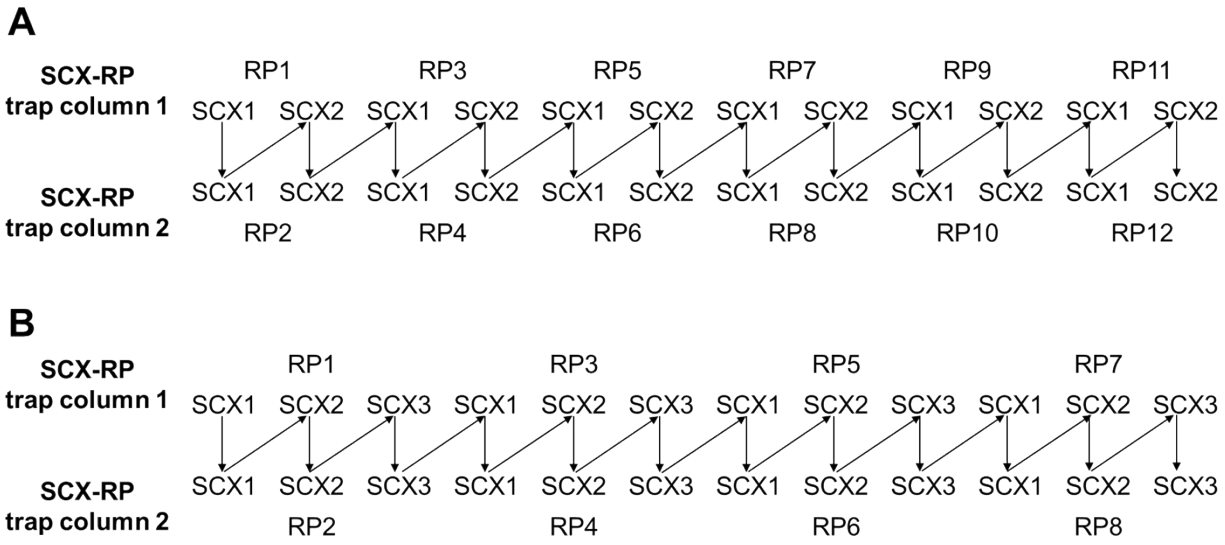


**Figure S-4.** Proportional Venn diagrams of (A) protein and (B) peptide identification obtained from the triplicate analysis of *Klebsiella oxytoca* lysate tryptic digests using 2D RP–RP.

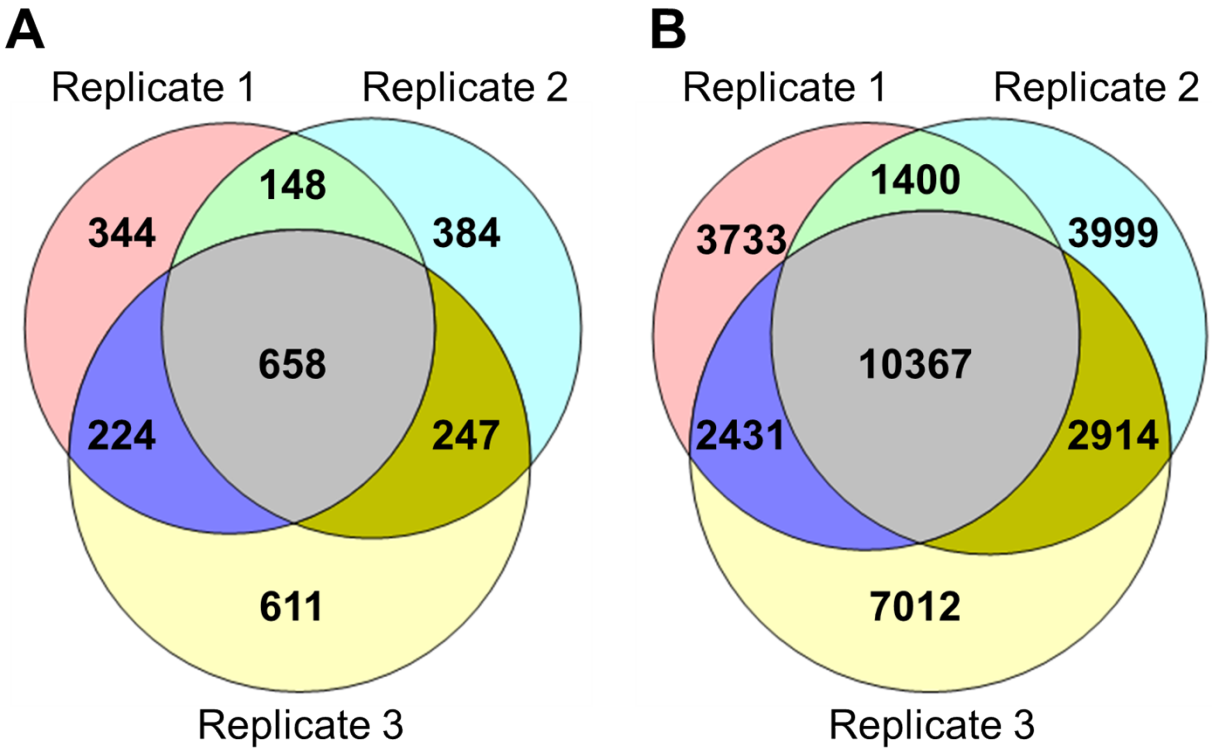
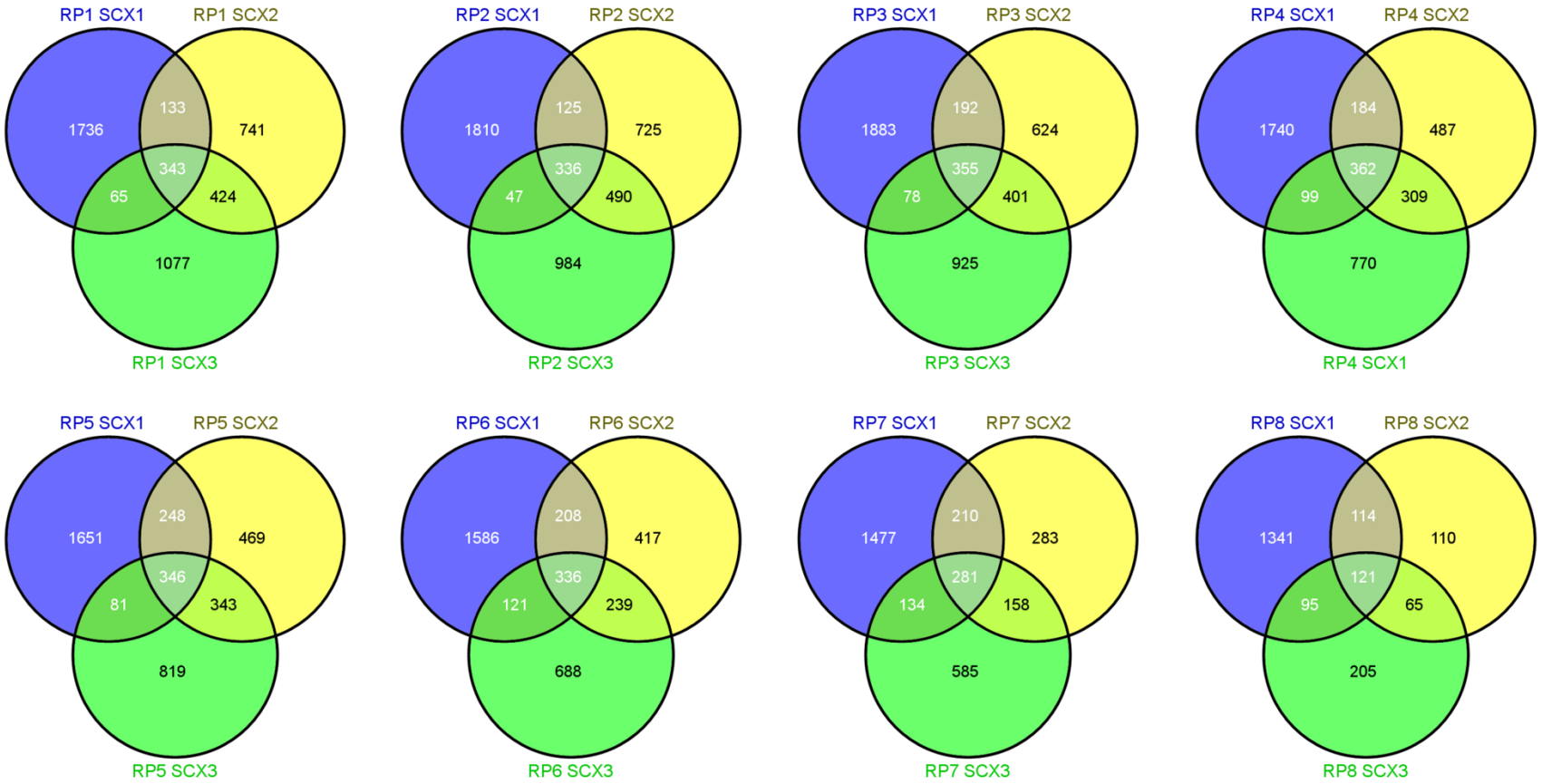


**Figure S-5.** Peptide spillover in SCX sub-fractions across the RP fractions in the analyses of mouse embryonic fibroblasts (STO) tryptic peptides with 3D RP–SCX–RP. The table below summarizes the percentage overlap of the peptides observed in the three SCX sub-fractions across the various RP fractions.



| RP fraction | % of peptide overlapping across 3 SCX sub-fractions |
|---|---|
| RP1 | 7.6% |
| RP2 | 7.4% |
| RP3 | 8.0% |
| RP4 | 9.2% |
| RP5 | 8.7% |
| RP6 | 9.3% |
| RP7 | 9.0% |
| RP8 | 5.9% |
| **Average** | **8.1%** |

**Figure S-6.** Mass spectra and chromatograms obtained during the elution of HFNAPSHIR$^{2+}$. Overlaid TOF survey scan during the elution of HFNAPSHIR$^{2+}$ from the (A) 3D RP–SCX–RP and (B) 2D RP–RP platforms. The red arrow indicates the position of the peptide peak of HFNAPSHIR$^{2+}$. The isotopic distribution of the peptide in the mass spectra after (C) 3D RP–SCX–RP and (D) 2D RP–RP separation. The extracted ion chromatogram of the peptide HFNAPSHIR$^{2+}$ after (E) 3D RP–SCX–RP and (F) 2D RP–RP separation. The MS/MS spectra of the peptide HFNAPSHIR$^{2+}$ acquired after (G) 3D RP–SCX–RP and (H) 2D RP–RP separation.

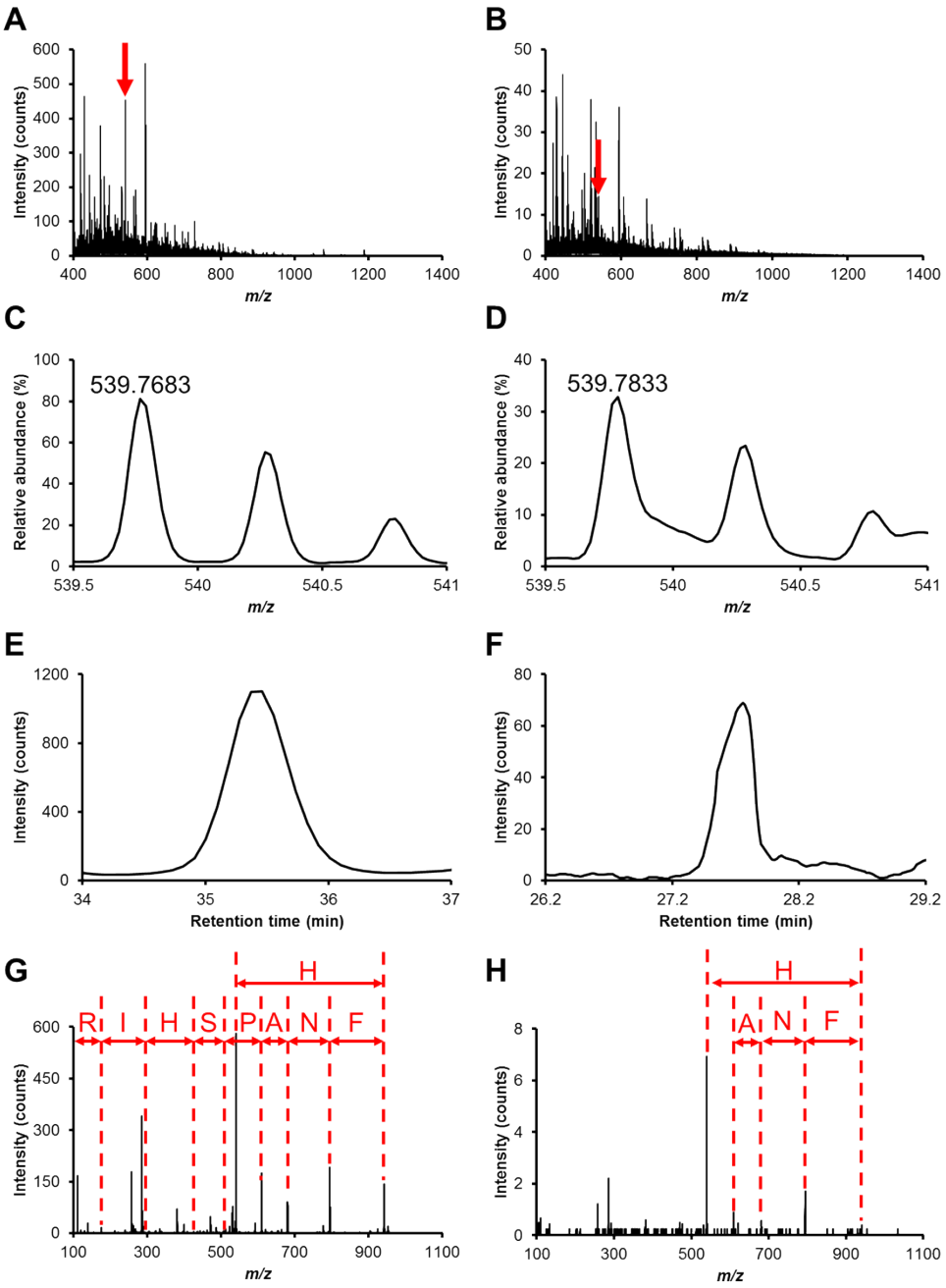


**Figure S-7.** Mass spectra and chromatograms obtained during the elution of TAVSHRPGAFK$^{2+}$. Overlaid TOF survey scan during the elution of TAVSHRPGAFK$^{2+}$ from the (A) 3D RP–SCX–RP and (B) 2D RP–RP platforms. The red arrow indicates the position of the peptide peak of TAVSHRPGAFK$^{2+}$. The isotopic distribution of the peptide in the mass spectra after (C) 3D RP–SCX–RP and (D) 2D RP–RP separation. The extracted ion chromatogram of the peptide TAVSHRPGAFK $^{2+}$ after (E) 3D RP–SCX–RP and (F) 2D RP–RP separation. The MS/MS spectra of the peptide TAVSHRPGAFK $^{2+}$ acquired after (G) 3D RP–SCX–RP separation.

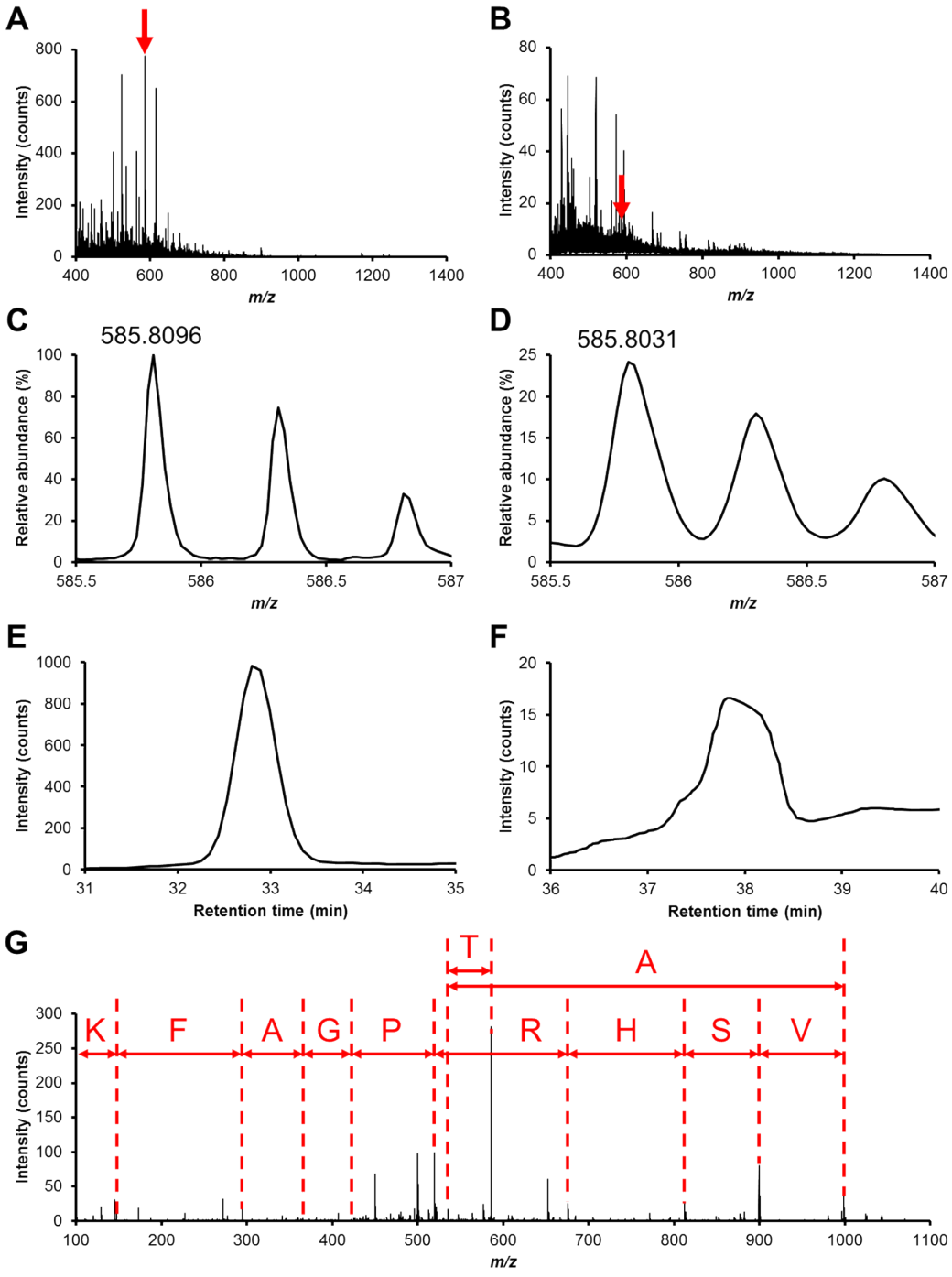


**Figure S-8.** Plot of p*I* with respect to molecular weight for the identified PC12 proteins, highlighting the unbiased protein profiling using the 3D RP–SCX–RP platform. Expected molecular weights and values of p*I* were determined using the Protein Digestion Simulator software at the OMICS.PNL.GOV website (http://omics.pnl.gov/software/ProteinDigestionSimulator.php). Proteins highlighted in red represent those at the extremes in terms of either p*I* and/or molecular weight for the sample population.

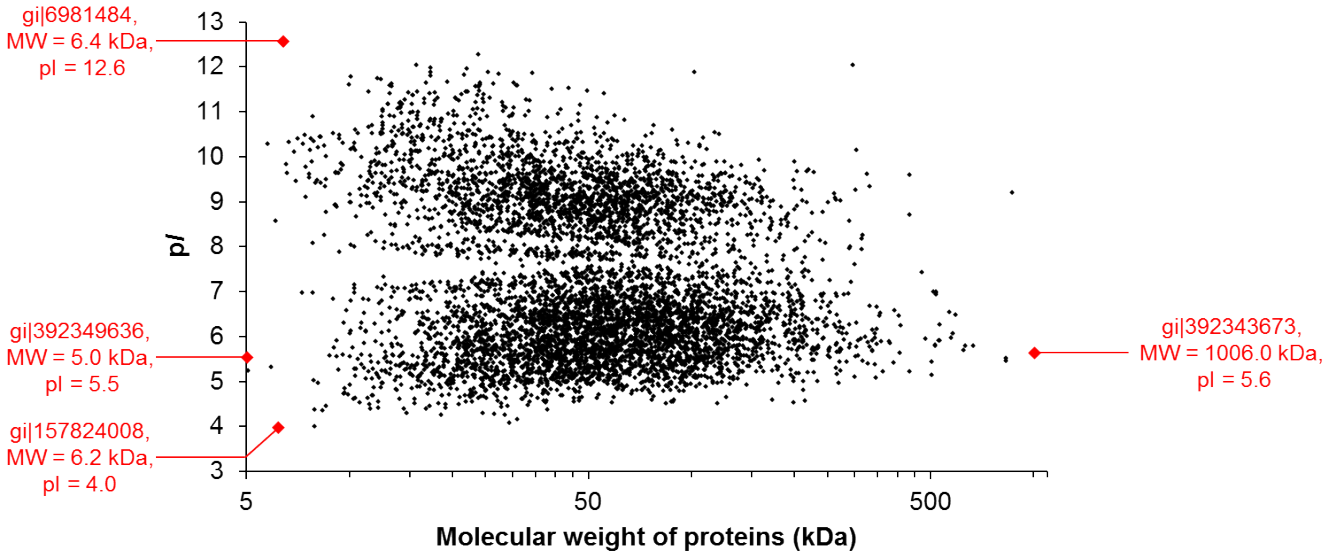


**Table S-1**. The division of gradients in the first dimension pH 10 RP for the 8, 12 and 16 fraction strategies used in 2D RP–RP and 3D RP–SCX–RP experiments. *The exact conditions of the gradient used in fractions RP 7-10 in the 16 fraction strategy were 16-17.5, 17.5-19, 19-20.5, 20.5-22 %B, respectively.

| 8 fraction | 12 fraction | 16 fraction | pH 10 RP %B |
|---|---|---|---|
| RP1 | RP1 | RP1 | 0-10 |
| | | RP2 | 10-12 |
| RP2 | RP2 | RP3 | 12-13 |
| | | RP4 | 13-14 |
| RP3 | RP3 | RP5 | 14-15 |
| | | RP6 | 15-16 |
| RP4 | RP4 | RP7* | 16-17 |
| | | | 17-18 |
| | RP5 | RP8* | 18-19 |
| RP5 | | RP9* | 19-20 |
| | RP6 | | 20-21 |
| | | RP10* | 21-22 |
| RP6 | RP7 | RP11 | 22-24 |
| | RP8 | RP12 | 24-26 |
| RP7 | RP9 | RP13 | 26-28 |
| | RP10 | RP14 | 28-30 |
| RP8 | RP11 | RP15 | 30-38 |
| | RP12 | RP16 | 38-46 |

**Table S-2** The final dimension pH 2 RP gradient conditions used in the (A) experiments with 8 pH 10 RP fraction strategy and 90 min gradient for the 2D RP–RP and 3D RP–SCX–RP platforms, (B) experiments with 12 pH 10 RP fraction strategy and 60, 90 or 180 min gradient for the 2D RP–RP or 3D RP–SCX–RP platforms and (C) experiments with 16 pH 10 RP fraction strategy and 45 min gradient for the 2D RP–RP.

**A**

| | pH 2 RP %D for the accumulative time point | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **90 min grad** | 0 min | 1 min | 91 min | 96 min | 106 min | 111 min | 116 min | 140 min |
| **RP1** | 0 | 8 | 20 | 20 | 50 | 50 | 0 | 0 |
| **RP2** | 0 | 8 | 25 | 25 | 50 | 50 | 0 | 0 |
| **RP3** | 0 | 10 | 25 | 25 | 50 | 50 | 0 | 0 |
| **RP4** | 0 | 10 | 28 | 28 | 50 | 50 | 0 | 0 |
| **RP5** | 0 | 12 | 30 | 30 | 50 | 50 | 0 | 0 |
| **RP6** | 0 | 12 | 32 | 32 | 50 | 50 | 0 | 0 |
| **RP7** | 0 | 15 | 35 | 35 | 50 | 50 | 0 | 0 |
| **RP8** | 0 | 20 | 40 | 40 | 80 | 80 | 0 | 0 |

**B**

| | pH 2 RP %D for the accumulative time point | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **60 min grad** | 0 min | 1 min | 61 min | 66 min | 76 min | 81 min | 86 min | 110 min |
| **90 min grad** | 0 min | 1 min | 91 min | 96 min | 106 min | 111 min | 116 min | 140 min |
| **180 min grad** | 0 min | 1 min | 181 min | 186 min | 196 min | 201 min | 206 min | 230 min |
| **RP1** | 0 | 8 | 20 | 20 | 50 | 50 | 0 | 0 |
| **RP2** | 0 | 8 | 20 | 20 | 50 | 50 | 0 | 0 |
| **RP3** | 0 | 10 | 22 | 22 | 50 | 50 | 0 | 0 |
| **RP4** | 0 | 10 | 25 | 25 | 50 | 50 | 0 | 0 |
| **RP5** | 0 | 12 | 25 | 25 | 50 | 50 | 0 | 0 |
| **RP6** | 0 | 12 | 28 | 28 | 50 | 50 | 0 | 0 |
| **RP7** | 0 | 15 | 30 | 30 | 50 | 50 | 0 | 0 |
| **RP8** | 0 | 15 | 32 | 32 | 50 | 50 | 0 | 0 |
| **RP9** | 0 | 15 | 35 | 35 | 50 | 50 | 0 | 0 |
| **RP10** | 0 | 18 | 35 | 35 | 50 | 50 | 0 | 0 |
| **RP11** | 0 | 20 | 35 | 35 | 50 | 50 | 0 | 0 |
| **RP12** | 0 | 20 | 35 | 35 | 80 | 80 | 0 | 0 |

**C**

| | pH 2 RP %D for the accumulative time point | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **45 min grad** | 0 min | 1 min | 46 min | 51 min | 61 min | 66 min | 71 min | 95 min |
| **RP1 – 2** | 0 | 8 | 20 | 20 | 50 | 50 | 0 | 0 |
| **RP3 – 4** | 0 | 8 | 25 | 25 | 50 | 50 | 0 | 0 |
| **RP5 – 6** | 0 | 10 | 25 | 25 | 50 | 50 | 0 | 0 |
| **RP7 – 8** | 0 | 10 | 28 | 28 | 50 | 50 | 0 | 0 |
| **RP9 – 10** | 0 | 12 | 30 | 30 | 50 | 50 | 0 | 0 |
| **RP11 - 12** | 0 | 12 | 32 | 32 | 50 | 50 | 0 | 0 |
| **RP13 – 14** | 0 | 15 | 35 | 35 | 50 | 50 | 0 | 0 |
| **RP15 – 16** | 0 | 20 | 40 | 40 | 80 | 80 | 0 | 0 |

**Table S-3.** Systematic name and name description of the 16 yeast proteins identified using 3D RP–SCX–RP, whose abundance is lower than 50 copies per cell as reported by Ghaemmaghami *et al*.[1]

| Systematic Name | Name Description |
|---|---|
| YBR006W | Succinate-semialdehyde dehydrogenase [NADP(+)] |
| YCL034W | LAS seventeen-binding protein 5 |
| YCL043C | Protein disulfide-isomerase |
| YDL204W | Reticulon-like protein 2 |
| YDR150W | Nuclear migration protein NUM1 |
| YGR152C | Ras-related protein RSR1 |
| YJL153C | Inositol-3-phosphate synthase |
| YJL167W | Farnesyl pyrophosphate synthase |
| YKL032C | Intrastrand cross-link recognition protein |
| YKL208W | Cytochrome b termination protein 1 |
| YLR100W | 3-keto-steroid reductase |
| YNL132W | UPF0202 protein KRE33 |
| YOR120W | Glycerol 2-dehydrogenase (NADP(+)) |
| YPR020W | ATP synthase subunit g, mitochondrial |
| YPR060C | Chorismate mutase |
| YPR148C | Uncharacterized protein YPR148C |

**Table S-4**. Functional significance of the unique yeast hydrophilic peptides identified using 3D RP–SCX–RP.

| Sequence | pH 2 HI value | Systematic Name | Name Description | Remarks[a] |
|---|---|---|---|---|
| HLIDS**VKL** | 10.18 | YAL054C | Acetyl-coenzyme A synthetase 1 | Peroxisomal targeting signal[2] |
| SV**DV**NNIQK | 14.23 | YKR080W | NAD(+) Methylenetetrahydrofolate dehydrogenase | NAD binding[3] |
| **SD**LAHLR | 14.55 | YER031C | GTP-binding protein YPT31/YPT8 | GTP binding[4, 5] |
| **VVSTGTSN**TATAGAVR | 15.53 | YJR144W | Mitochondrial genome maintenance protein MGM101 | Mitochondrial targeting signal[6-8] |
| **FTKPTPVQ**K | 15.59 | YOR204W | ATP-dependent RNA helicase DED1 | Q motif, adenine recognition and ATPase activity[9, 10] |
| **WAQDQVTK** | 16.23 | YDR129C | Fimbrin | Actin binding site 3-2[11] |
| **T**VISQSLSK | 17.99 | YDL185W | V-type proton ATPase catalytic subunit A | ATP binding[12] |
| **K**GETLQDTIR | 18.12 | YJL130C | Carbamoylphosphate synthetase-aspartate transcarbamylase | Aspartate transcarbamylase domain catalytic site[13, 14] |

[a] denotes functional significance of the bolded and underlined residues in the identified peptides. The representative peptides are listed in descending order according to their predicted pH 2 hydrophobicity index values.

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