<Supporting Information>

Anion and Cation Mixed-Bed Ion Exchange for Enhanced Multidimensional Separations of Peptides and Phosphopeptides

*Akira Motoyama, Tao Xu, Cristian I. Ruse, James A. Wohlschlegel and John R. Yates, III**

Department of Cell Biology

The Scripps Research Institute

10550 North Torrey Pines Road, La Jolla, CA 92037, USA

jyates@scripps.edu

Table of Contents

1. Figure S-1
2. Figure S-2
3. Figure S-3
4. Figure S-4
5. Figure S-5

Figure S-1

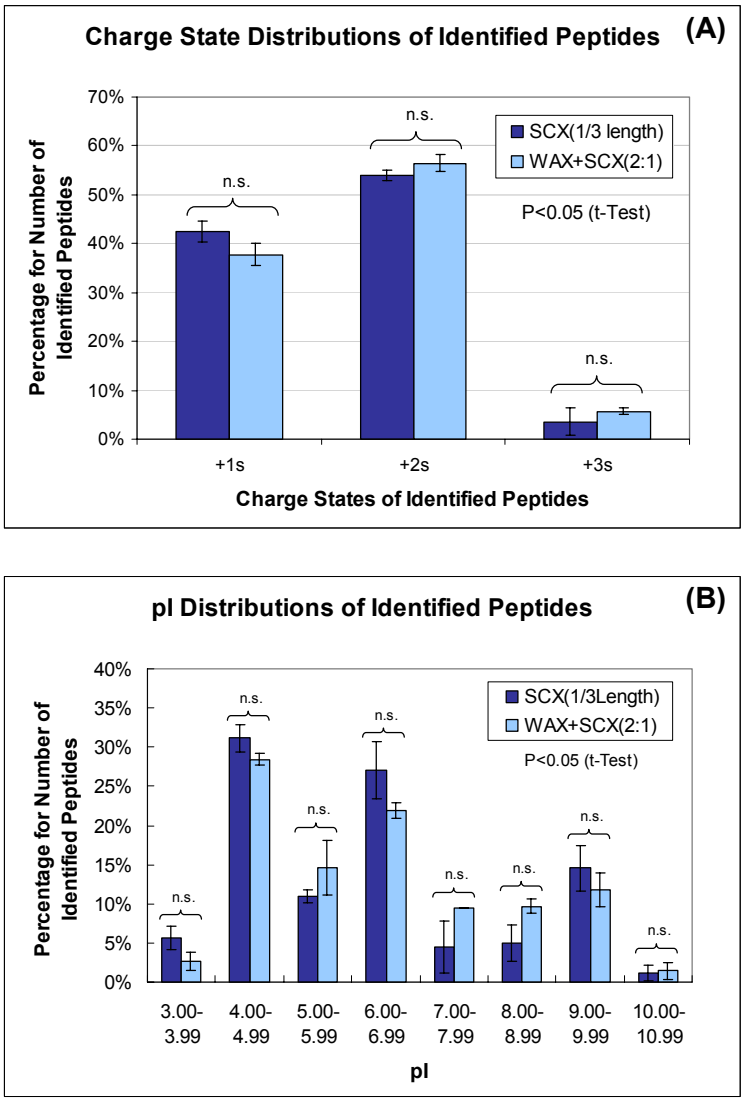


Figure S-1 **Comparison of charge state distribution (A) and p*I* distribution (B) of identified peptides in SCX and ACE mixed-bed formats**

The triplicate datasets from the recovery experiments for both SCX and ACE mixed-bed were statistically compared by t-Test under an equal variance assumption. The total number of peptides used for the charge state distribution analysis (A) was 290 and 406 for SCX(1/3 length) and WAX+SCX(2:1), respectively. For the p*I* distribution analysis (B), 235 and 328 peptides were analyzed for SCX(1/3 length) and WAX+SCX(2:1), respectively. The difference in their total peptide numbers resulted from the identification of peptides from multiple charge states that were considered in the p*I* distribution analysis. In both comparisons, there were no statistically significant differences observed.

Figure S-2

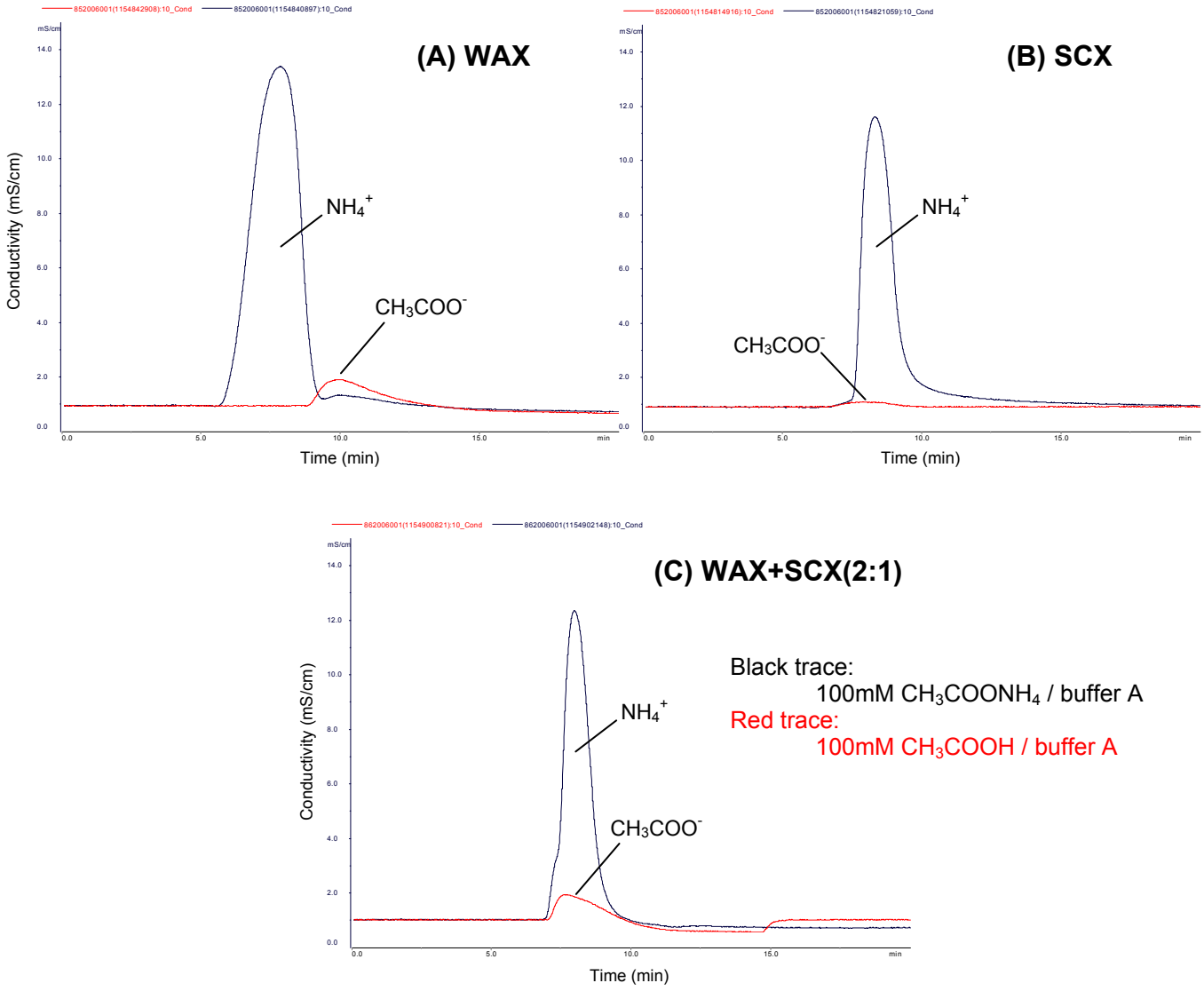


Figure S-2 **Separation of salt cations/anions during salt pulse: comparison between columns packed with WAX (A), SCX (B), and their 2:1 mixture (C)**

A two-minute equivalent of a salt buffer pulse was applied to each ion-exchanger column directly connected to an Ettan MDLC system (GE Healthcare, Pittsburgh, PA) equipped with a conductivity detector. The salt pulse solutions applied were 100 mM ammonium acetate/buffer A (for the black trace) and 100 mM acetic acid/buffer A (for the red trace). The packing materials, column dimensions, mobile phase, and column flow rate were identical to those of the other experiments in the study (column: 0.25 mm I.D. x 2.5 cm, flow rate: 300~330 nL/min (split from 250 μL/min pump flow rate), detection: conductivity, mobile phase: buffer A (acetonitrile-water-formic acid (5:95:0.1, v/v/v))). The salt pulse was generated by a 500 μL sample loop placed in between the HPLC pump and the ion-exchanger column (before the splitter). The column flow rate was monitored by a micro flow meter throughout each run.

Figure S-3

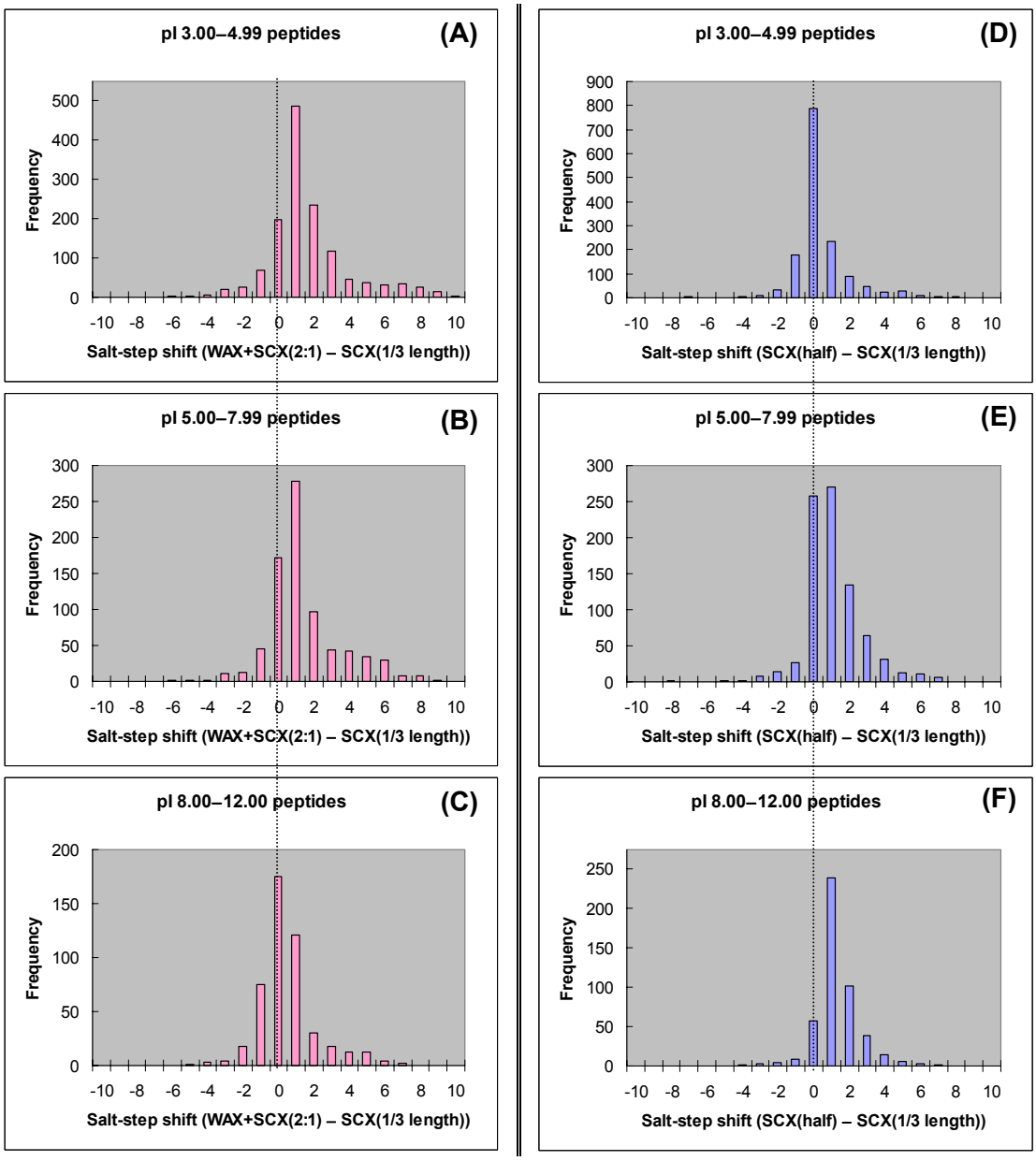


Figure S-3 **Comparison of salt-step shift of commonly identified peptides in two ion-exchanger formats ((A)-(C): (WAX+SCX(2:1))–(SCX), (D)-(F): (SCX(half length))–(SCX (1/3 length)))**

Peptides identified in common between two ion-exchanger formats were first divided into three p*I* groups (3.00-4.99, 5.00-7.99, and 8.00-12.00). For each group, the salt step information at which the peptide was first identified in the 13-step MudPIT was compared. The left panel ((A)-(C)) is a comparison between WAX+SCX(2:1) and SCX, while the right panel ((D)-(F)) is that of SCX(half length) and (SCX (1/3 length). For example, if a peptide was identified in the same salt step between the two different ion-exchanger formats being compared, the peptide is counted in the bin "salt-step shift = 0". Likewise, the bins having + "salt-step shifts" consist of peptides exhibiting stronger retention in the

ion-exchanger to be subtracted (WAX+SCX(2:1) or SCX(half length) in the above case). Data was obtained with an LTQ-Orbitrap using high mass-accuracy precursor measurement in order to have high confidence on 1-peptide hit assignments. The measurements were taken after external calibration with a typical mass error of less than 5 ppm. The precursor mass tolerance to identify peptides was set to 10 ppm and the false positive rate was set to less than 0.05% at spectrum level. The analyzed samples, columns used, and other chromatographic conditions were same as those of Figure 5.

<Mass Spectrometry Conditions>

The LTQ-Orbitrap was operated in a parallel scan mode where one full MS scan in the Orbitrap at 6,000 resolution is collected simultaneously with 4 tandem MS scans in the LTQ. The mass range for the full MS scan was *m/z* 400-2,000, and the normalized collision energy for tandem MS was set to 35%. The Orbitrap was calibrated before each run with the standard calibration solution suggested by the manufacturer. No post-data-processing such as re-calibration was conducted. The number of microscans was set to 1 for both MS and MS/MS scans. The dynamic exclusion settings used were as follows: repeat count: 1, repeat duration: 0.50 min, exclusion list size: 150, and exclusion duration: 3.00 min.

<Data Analysis>

Collected MS/MS spectra were processed using the same procedure described in the Experimental Procedure section except that a modified version of SEQUEST was used to circumvent the problem that monoisotopic masses cannot be accurately determined from precursor ions in which the full isotopic packet is not observed. The modified SEQUEST was developed in house to take into account the fact that the ion that triggered the data-dependent MS/MS scan may not be the monoisotopic ion but a different isotopomer and divides the search into small *m/z* windows centered on the predicted isotopomer masses. With this feature, a mass tolerance of 20 ppm for the precursor peptide was used in the SEQUEST database search. Cysteine residues were considered to have a static modification of + 57.02146 mass units. Only half- or fully tryptic peptides were accepted to identify peptides/proteins. The database search was performed without enzyme specificity.

Figure S-4

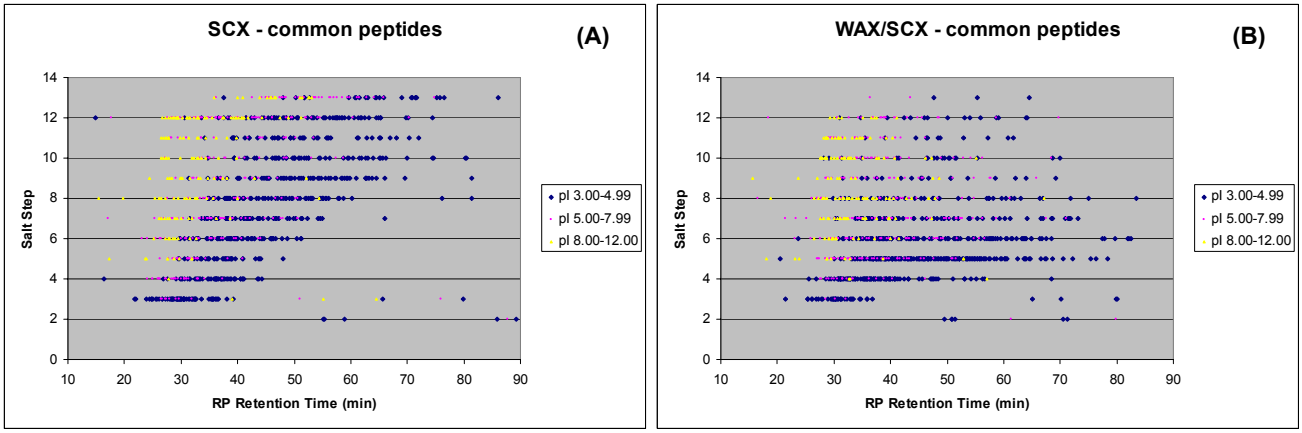
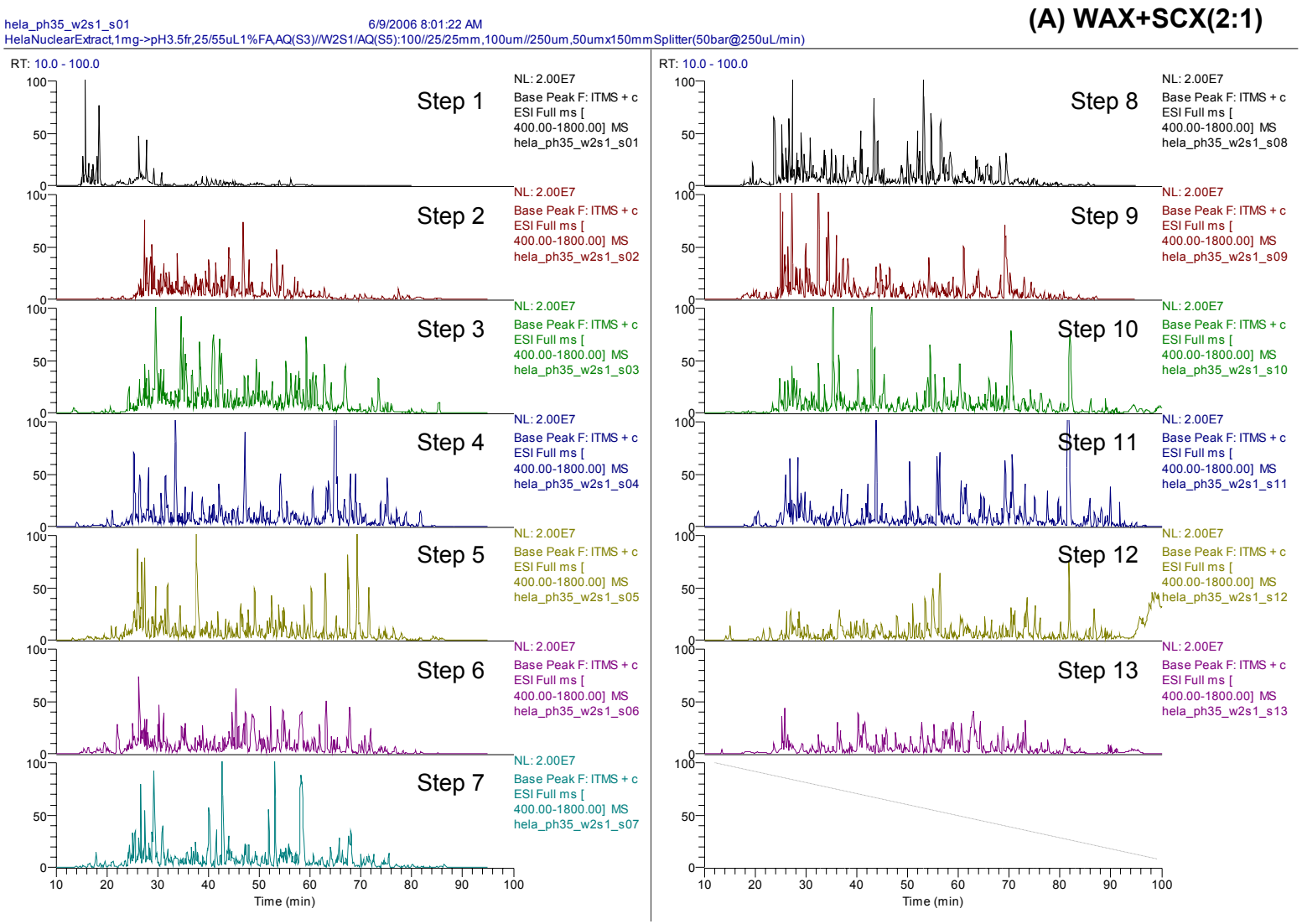


Figure S-4 Comparison on the distribution of peptides commonly identified in SCX and WAX/SCX combinations

The distribution of commonly identified peptides in the two formats (SCX and WAX/SCX) in 2D planes that consisted of salt step in which the peptide eluted and its RP retention time. The plots were generated in the same way as Figures 5 (D) and (F) except that only the commonly identified peptides were displayed.

Figure S-5


hela_ph35_w2s1_s01 6/9/2006 8:01:22 AM
HelaNuclearExtract,1mg->pH3.5fr,25/55uL1%FA,AQ(S3)//W2S1/AQ(S5):100//25/25mm,100um//250um,50umx150mmSplitter(50bar@250uL/min)
(A) WAX+SCX(2:1)
RT: 10.0 - 100.0
Step 1
Step 2
Step 3
Step 4
Step 5
Step 6
Step 7
Step 8
Step 9
Step 10
Step 11
Step 12
Step 13
NL: 2.00E7
Base Peak F: ITMS + c ESI Full ms [400.00-1800.00] MS hela_ph35_w2s1_s01
Base Peak F: ITMS + c ESI Full ms [400.00-1800.00] MS hela_ph35_w2s1_s02
Base Peak F: ITMS + c ESI Full ms [400.00-1800.00] MS hela_ph35_w2s1_s03
Base Peak F: ITMS + c ESI Full ms [400.00-1800.00] MS hela_ph35_w2s1_s04
Base Peak F: ITMS + c ESI Full ms [400.00-1800.00] MS hela_ph35_w2s1_s05
Base Peak F: ITMS + c ESI Full ms [400.00-1800.00] MS hela_ph35_w2s1_s06
Base Peak F: ITMS + c ESI Full ms [400.00-1800.00] MS hela_ph35_w2s1_s07
Base Peak F: ITMS + c ESI Full ms [400.00-1800.00] MS hela_ph35_w2s1_s08
Base Peak F: ITMS + c ESI Full ms [400.00-1800.00] MS hela_ph35_w2s1_s09
Base Peak F: ITMS + c ESI Full ms [400.00-1800.00] MS hela_ph35_w2s1_s10
Base Peak F: ITMS + c ESI Full ms [400.00-1800.00] MS hela_ph35_w2s1_s11
Base Peak F: ITMS + c ESI Full ms [400.00-1800.00] MS hela_ph35_w2s1_s12
Base Peak F: ITMS + c ESI Full ms [400.00-1800.00] MS hela_ph35_w2s1_s13
Time (min)

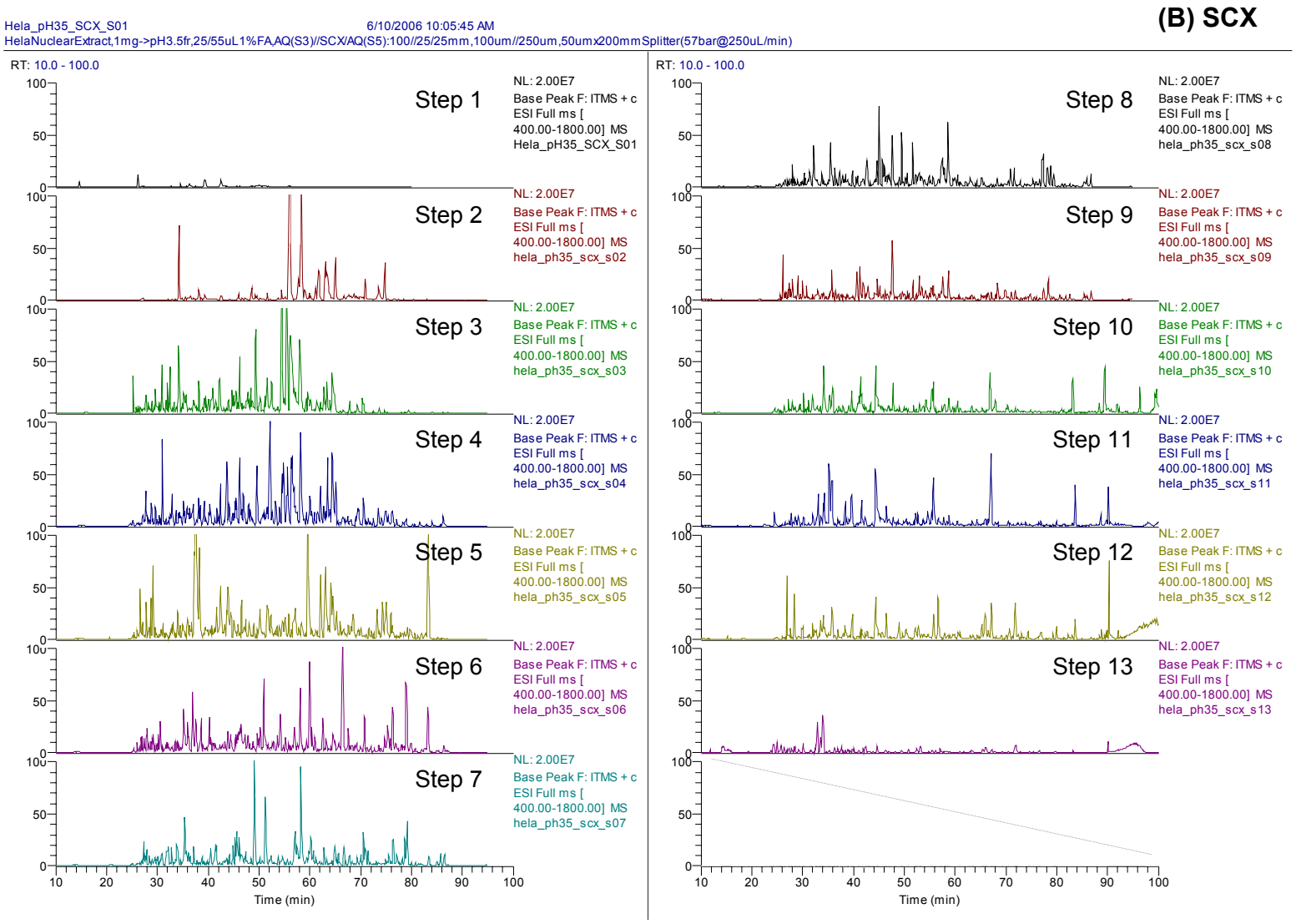


Figure S-5 Base peak chromatograms of 13-step MudPIT runs of phosphopeptide-enriched samples[30] analyzed by WAX+SCX(2:1) (A) and SCX (B) formats

Approximately 50 μg of tryptic digests (phosphopeptide-enriched samples, pH 3.5 fraction[30]) were analyzed by 13-step MudPIT runs using an LTQ mass spectrometer. The LTQ was programmed to conduct neutral-loss triggered data-dependent MS/MS for the peptides that

produced 49 or 98 amu neutral losses from their precursor ions. The y-axis is normalized throughout all chromatograms for comparison. The results from these runs were used to generate Figure 6 and Table 1.