

Chapter 12

Offline High pH Reversed-Phase Peptide Fractionation for Deep Phosphoproteome Coverage

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Abstract

Protein phosphorylation, a process in which kinases modify serines, threonines, and tyrosines with phosphoryl groups is of major importance in eukaryotic biology. Protein phosphorylation events are key initiators of signaling responses which determine cellular outcomes after environmental and metabolic stimuli, and are thus highly regulated. Therefore, studying the mechanism of regulation by phosphorylation, and pinpointing the exact site of phosphorylation on proteins is of high importance. This protocol describes in detail a phosphoproteomics workflow for ultra-deep coverage by fractionating peptide mixtures based on high pH (basic) reversed-phase chromatography prior to phosphopeptide enrichment and mass spectrometric analysis. Peptides are separated on a C₁₈ reversed-phase column under basic conditions and fractions collected in timed intervals followed by concatenation of the fractions. Each Fraction is subsequently enriched for phosphopeptides using TiO₂ followed by LC/MS analysis.

Key words Phosphoproteomics, High pH, Reversed-phase, Phosphorylation, Phosphopeptide enrichment, Fractionation

1 Introduction

Protein phosphorylation is a pivotal post-translational modification involved in regulating virtually all cellular processes. Site-specific phosphorylation often functions as a cellular switch, which can alter the activity of a protein, change its subcellular localization or interaction partners, as well as mark proteins for degradation [1]. The temporal and spatial distribution of protein phosphorylation in a cell is controlled by the action of protein kinases and counteracted by protein phosphatases in an interplay that is tightly regulated in healthy cells under physiological conditions [2–4]. However, misregulation of this cellular control as a result of genomic mutations, DNA damage, and other factors often leads to diseases such as cancer [4]. Understanding the regulation and dynamics of protein phosphorylation in health and disease is therefore of great importance. Although many different analytical

strategies exist for investigation of the phosphoproteome, enrichment and fractionation is usually required for deep coverage. This is due to the high dynamic range and complexity of eukaryotic phosphoproteomes as well as generally low stoichiometry of phosphorylation sites and hence low abundance of phosphopeptides in total cell digests. We recently described an offline peptide fractionation method as a powerful tool to improve depth and coverage in phosphoproteome analyses [5]. The method relies on fractionating tryptic peptides on a hydrophobic C₁₈ column under basic conditions (pH > 8). Although several methods and strategies exist for fractionating peptides, high pH reversed-phase fractionation has shown great promise in recent years due to its high separation and resolving power compared to standard methods such as those based on ion exchange or hydrophilic interaction chromatography [6]. Fractionation based on high pH reversed-phase requires concatenation of fractions from different parts of the elution gradient to be orthogonal with downstream low pH reversed-phase separation in tandem with mass spectrometry analysis [7]. Following collection and concatenation of fractions, phosphopeptides are enriched using titanium dioxide beads [8]. Depending on starting amounts, it is possible to map close to 40,000 phosphopeptides within 24 h of mass spectrometric analysis time using this technique.

2 Materials

All buffers are made with sequencing grade chemicals and ultra-pure water (Milli-Q).

2.1 Cell Lysis and Digestion

1. Eukaryotic cells (adherent or suspension) grown in large amounts (*see Note 1*).
2. Cell lysis buffer such as RIPA, urea, or guanidinium hydrochloride (*see Note 2*).
3. Acetone.
4. Bradford or other protein concentration determination assay.
5. Reducing reagents such as tris(2-carboxyethyl)phosphine (TCEP) or dithiothreitol (DTT) (*see Note 3*).
6. Alkylation reagent chloroacetamide (CAA).
7. Lys-C and trypsin.

2.2 Buffers for Fractionation

1. Tryptic digested peptide mixture in basic condition (*see Note 4*).
2. BSA or other peptide standard mixture.
3. Buffer A, 5 mM ammonium hydroxide (*see Note 5*).
4. Buffer B, 90 % acetonitrile, 5 mM ammonium hydroxide.
5. Optional Buffer C for compatible LC systems (*see Note 6*).

2.3 Fractionation and Concatenation

1. High pH Compatible C₁₈ reversed-phase column, 4.6 mm ID × 250 mm (*see Note 7*).
2. High performance liquid chromatography (HPLC) system compatible up to 400 bars (5800 psi) with auto sampler, UV detector, pressure sensor, and fraction collector (*see Note 6*) is recommended.
3. 96 deep well plates.

2.4 Phosphopeptide Enrichment and STAGE Tipping

1. Titanium dioxide (TiO₂) beads.
2. 2,5-dihydroxybenzoic acid (20 mg/ml) in 80 % ACN, 1 % TFA.
3. C8 STAGE tip and C18 STAGE tip.
4. Trifluoroacetic acid (TFA).
5. 5 % ammonium hydroxide (NH₄OH).
6. 20 % ammonium hydroxide (NH₄OH), 25 % acetonitrile.

2.5 Nano-Liquid Chromatography (nLC) and Mass Spectrometer (MS)

1. Capillary (75 μm internal diameter, 15 cm length) column packed with 3 μm or smaller C₁₈ beads.
2. Nano-LC system compatible up to 280 bars (for 15 cm column), coupled to MS.
3. High performance mass spectrometers capable of MS analysis at high resolution (>10,000) such as Orbitrap Q-Exactive (Thermo Fischer) or time of flight instruments such as 5600 Q-TOF (AB Sciex).

3 Methods

3.1 Cell Lysis and Protease Trypsin Digestion

1. Lyse cells using RIPA, urea or guanidinium hydrochloride (*see Note 2*).
2. If using RIPA buffer, acetone precipitate proteins, centrifuge to remove acetone and resuspend protein pellet in urea or guanidinium hydrochloride.
3. Determine protein concentration using Bradford or other protein assay.
4. Reduce proteins with DTT (1 mM) or TCEP (5 mM) for 30 min at RT.
5. Alkylate cysteines with CAA (5 mM) at RT in the dark. This can be done simultaneously in combination with TCEP (*see Note 3*).
6. Add Lys-C (1:100 protease–protein ratio) at room temperature for 4 h.
7. Dilute urea to >2 M (>1 M for guanidinium hydrochloride) with 50 mM ammonium bicarbonate buffer.
8. Add trypsin (1:50 protease–protein ratio), digest overnight at 37 °C.

3.2 *Sample Preparation for Fractionation*

1. Tryptic peptide mixture should be prepared and desalted on reversed-phase C₁₈ cartridges such as C₁₈ sep-pak to remove salts prior to fractionation. Cell lysis additives (such as detergents), which can interfere with fractionation and other downstream analysis must be removed.
2. Speedvac peptide mixture to evaporate acetonitrile (*see Note 8*).
3. Add Buffer A to the sample to bring it to roughly same starting condition (i.e., pH and mobile phase additives such as ammonium hydroxide similar to running buffers) as LC. Sample volume should be kept below the injection maximum of the LC system autosampler and injection loop.
4. Prepare BSA peptide mixture in a similar fashion.

3.3 *High Pressure Liquid Chromatography Preparation (HPLC)*

1. Prepare column by connecting to HPLC system.
2. Depending on the internal diameter of the column and manufacturer instructions, run Buffer A with recommended optimal flow rate (*see Notes 6 and 9*).
3. Monitor peptide elution by UV at absorbance 214 nm wavelength, which is specific for peptide bonds. Absorbance at 260 and 280 nm may also be used however they are not as accurate.
4. Monitor column pressure over time so that it reaches a stable baseline (*see Note 9*).
5. Maintain flow rate on the column until a stable UV baseline is observed and expected column pressure is achieved. This may take several minutes depending on column condition and equilibration (*see Note 9*).

3.4 *Gradient Design*

Please note that elevated column temperatures for separation at high pH are detrimental for column bed material. We therefore recommend performing fractionation at ambient temperatures (~25 °C). *See Fig. 1* for example of typical gradient used for fractionation.

1. Design a gradient starting from low Buffer B composition (0–5 %) to 25 % in 60–90 min (*see Note 10*).
2. The next part of the gradient should go from 25 to 40 % Buffer B in 5–10 min.
3. Add a quick ramp up to 60–70 % B in 5 min. At this point the fraction collection can be stopped.
4. Maintain gradient at high Buffer B for up to 5 min before ramping back down to 0–5 % Buffer B.
5. Hold at low Buffer B for 5–10 min to allow for column re-equilibration.

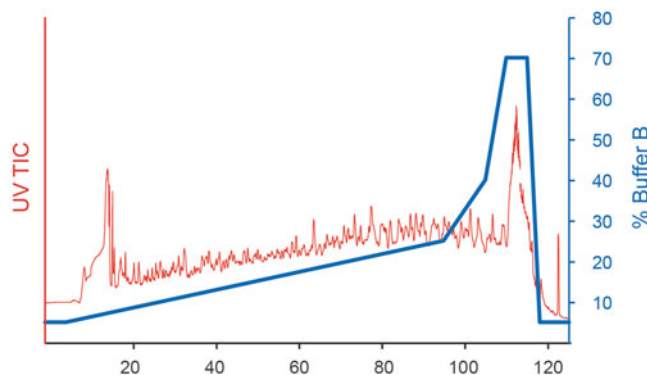


Fig. 1 Fractionation gradient of Buffer B concentration is overlapped with the UV total ion chromatogram (TIC)

3.5 Determining Column Condition

Before and after fractionating peptide mixtures at high pH, it is recommended to determine the condition of the column using a standardized peptide mixture. Steps for determining column condition and separation capacity using bovine albumin (BSA) peptide mixture are recommended below. Each run should be monitored using UV absorbance and pressure. BSA sample does not need to be fraction collected and can be sent to waste.

1. Design gradient using buffers without ammonium hydroxide for analysis of BSA peptides. Start from 5 % B to 30 % B in 20–30 min.
2. Ramp B from 30 to 40 % in 5 min.
3. Ramp B from 40 to 60 % in 2 min, hold for 2–5 min.
4. Decrease B from 60 to 5 % in 2 min.
5. Maintain at 5 % B for 5–10 min.
6. Inject 10–100 μg of BSA peptides onto column using described gradient.
7. Determine baseline and peak conditions using UV trace (*see Note 11*).

3.6 Sample Injection and Fraction Collection

Please note that it is recommended to first run a BSA mixture prior to running fractionating on a new column (*see Subheading 3.5*).

1. Prepare sample for injection onto the column using auto-sampler or with an additional line (*see Note 12*). Inject volume based on sample concentration and column capacity (roughly 5–8 mg for 4.6 mm ID \times 250 mm C_{18} columns).
2. Run program with gradient described in Subheading 3.3.

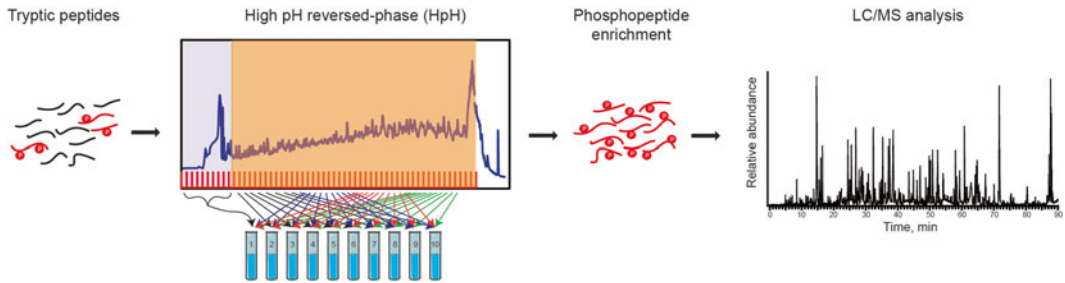


Fig. 2 Basic workflow for fractionation with high pH reversed-phase is presented. Tryptic peptides are fractionated on a reversed-phase C_{18} column using high pH buffers. The fractions are then concatenated to mix different parts of the gradient so they are orthogonal to downstream LC/MS analysis at low pH. Fractions are then enriched for phosphopeptides and analyzed via LC/MS

3. Collect fractions every 30–60 s in a deep well plate or Eppendorf tubes, depending on fraction collector and rack type (*see Note 13*).
4. Store samples at 4 °C or proceed to next step (samples can be stored for up to 2 weeks).

3.7 Fraction Concatenation

1. Prepare samples for concatenation by first pooling flow-through region as one pre-concatenated fraction (*see Fig. 2*).
2. Concatenate to 10–15 fractions (*see Figs. 2 and 3*).
3. Although not necessary some fractions such as the first fraction have larger volumes that can be decreased by drying in a speed-vac (*see Note 14*).

3.8 Post Fractionation Column Care

In order to extend the life of the fractionation column some steps are recommended below (*see Note 15*).

1. After running the fractionation gradient a post wash method is recommended with buffers containing no ammonium hydroxide and only water and acetonitrile as Buffers A and B.
2. Design a wash gradient which ramps Buffer B (acetonitrile) from 5 to 90 % in 15–20 min where it should be held for 5–10 min.
3. Ramp B down to 5 % in 5 min.
4. Repeat **steps 2 and 3**.
5. End step with 50–70 % B running at low flow rates (0.05–0.100 $\mu\text{l}/\text{min}$) where the column can sit idle. If column is not to be used for long periods of time it is recommended to cap and store the column (*see next step*).
6. Cap column from one side and let pressure build up slightly while low flow is still running.
7. Quickly remove column from the other side and cap column for storage.

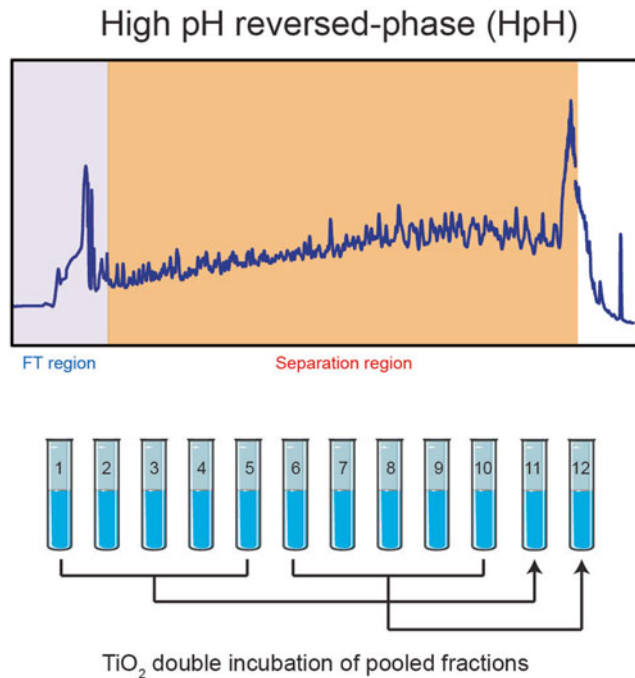


Fig. 3 Strategy for double incubation of fractions with TiO₂ beads is presented. After each fraction is enriched for phosphopeptides, the supernatants are pooled together for double TiO₂ enrichment

3.9 Preparing Fractions for Phosphopeptide Enrichment

The following steps are an outline for preparation of fractions for enrichment of phosphopeptides with titanium dioxide (TiO₂) beads.

1. Measure peptide concentration of each fraction using A_{280} nanodrop or other estimation methods compatible with peptides such as Lowry assay.
2. Bring the fractionated peptide solution to conditions compatible with enrichment method. For TiO₂ enrichment, add acetonitrile to 60–80 % and trifluoroacetic acid (TFA) to a final concentration of 5–6 %.
3. Samples may also be stored in this condition at $-20\text{ }^{\circ}\text{C}$ for several days up to a few weeks.

3.10 Enrichment of Phosphopeptides and Preparation for LC/MS Analysis

We recommend fractionating peptide mixtures prior to enrichment of phosphopeptides. The resultant reduction in sample complexity leads to more efficient enrichment of phosphopeptide mixtures and thus deeper coverage. The method described below is for enrichment using titanium dioxide beads (MOAC), please consult

manufacturer protocols for different enrichment strategies such as IMAC.

1. Weigh TiO_2 beads (1:2 peptide–bead ratio) and resuspend in DHB buffer (we recommend 10 μl DHB buffer per milligram of TiO_2 beads). Incubate for 20 min while rotating or shaking.
2. Add TiO_2 bead solution to the fractionated peptide solution at a ratio of 2 mg beads (20 μl of TiO_2 —DHB solution in this case) per milligram of peptides.
3. Incubate fractions with TiO_2 beads for 30 min at room temperature on a sample rotator.
4. Centrifuge fractions to form a pellet with TiO_2 beads. Do not discard supernatant if performing proteomics analysis. Also keep the supernatant if performing another enrichment round to recover multiphosphorylated peptides as described in Subheading 3.9, steps 6 and 7.
5. Resuspend pellet with 100 μl of 50 % acetonitrile and 5 % TFA.
6. After centrifugation pool supernatants together into 1–3 fractions (*see* Fig. 2).
7. Perform Subheading 3.9, steps 2–5 on the pooled fractions to enrich pooled fractions again for phosphopeptides leading to a higher recovery of multiply phosphorylated peptides.
8. Load beads suspended in 100 μl of 50 % acetonitrile and 5 % TFA on C_8 STAGE tip.
9. Centrifuge 3 min at $800\times g$ to remove buffer followed by washing with 30 % acetonitrile, 1 % TFA (add additional centrifugation time, if not all the buffer has washed through the STAGE tip).
10. Centrifuge to remove buffer followed by washing with 60 % acetonitrile with 1 % TFA. Elute with 20 μl 5 % NH_4OH , followed by 20 μl of 20 % NH_4OH with 25 % acetonitrile into a new tube.
11. Evaporate (not to dryness) samples in speedvac for 15 min at 45 °C.
12. Resuspend samples in acidic buffer (1 % TFA, 2 % acetonitrile).
13. Prepare C_{18} STAGE tip by washing/centrifuging for 2 min at $800\times g$ each with methanol (20 μl), 80 % acetonitrile with 0.1 % TFA (20 μl), 3 % acetonitrile, 0.1 % TFA (40 μl). Last wash might require additional centrifugation time.
14. Load samples on C_{18} STAGE tip, centrifuge at $800\times g$ until the sample is washed through and wash with 3 % acetonitrile with 1 % TFA (samples can be stored in this condition).
15. Elute samples with buffers containing 40–60 % acetonitrile, 0.1 % TFA. Speedvac and resuspend in Buffer A.

3.11 LC/MS

Prior to LC/MS analysis it is important to have a method ready for phosphoproteome analysis. The details described below are for a Q-Exactive mass spectrometer coupled to a nanoLC system. For different systems we recommend consulting manufacturer settings or published literature for guidance. For phosphopeptide analysis, we have modified the method for sensitive analysis; this is due to the fact that sample complexity is reduced in fractionated and phospho-enriched samples where quality of the fragment HCD (high collisional dissociation) spectrum is favored over sequencing speed [9].

1. Design a LC method with nLC gradient of 60 min or more. If not limited by instrument time we recommend 120 min.
2. Include ramp up to 80 % Buffer B (80 % acetonitrile) and back down to 5 % B (starting conditions of LC method).
3. Injection volume should be kept to 5 μ l or below for each sample (*see Note 16*).
4. Design a top 10 method on the Q-Exactive with 70,000 MS resolution and 35,000 dd-ms2 resolution.
5. The scan range for the MS should be set to 400–1600 m/z , for dd-ms2 fixed first mass should be set to 100 m/z .
6. Maximum injection time for the MS should be 20 ms, injection time for dd-ms2 should be 108 ms so it is in parallel with the Orbitrap transient at 35,000 resolution.
7. Automatic gain control AGC target for MS should be set to $1e6$ or higher, for dd-ms2 it should be $10\times$ lower (100,000 in this case).
8. Quadrupole isolation window (width) should be set to 1 m/z (but not lower)—2 m/z (not higher).
9. Normalized collisional energy should be set to a value between 25 and 30 (should be higher for phosphopeptides), we recommend 28 as safe median.
10. Dynamic exclusion should be set to 30 s.
11. Run samples with this method.

3.12 Data Analysis

After raw files have been collected, use LC/MS data analysis software of your choice. It is recommended to use software packages which can perform data analysis for post-translational modifications. Software packages such as MaxQuant (Mann Lab, www.maxquant.org), Mascot (Matrix Science), and Proteome Discoverer (Thermo Scientific) are good options. When performing analysis with the software package it is important to specify phosphorylation modification of serine, threonine, and tyrosine as variable modifications. Additionally, we also recommend the addition of protein acetyl-n terminus, methionine oxidation as variable modification. Since cysteines are modified to completion using

alkylating reagent (CAA in our case) it is important to set that as a fixed modification (carbamidomethylation for CAA). If using a different alkylating reagent, it is important to specify the correct fixed modification. When using high resolution mass spectrometers, it is important to limit the parts per million tolerance at the MS and MS/MS levels in order to increase the confidence for peptide hits. This is important for PTM analysis where proper localization and peptide sequence coverage of the modification is very important for site localization. When searching against the FASTA database of your organism, it is crucial that peptide false discovery rate is set to 1 % or lower. Vendor instructions for each software packages as well as published literature can aid in selecting proper parameters for each software package [10].

4 Notes

1. The shown experiments were done with tryptic digests of whole cell lysates from mammalian HeLa cervix carcinoma cells. Cells were serum starved overnight in DMEM and stimulated for 10 min with serum prior to cell lysis.
2. There are various options available for lysing cells. Lysis buffer containing detergents such as SDS require additional steps for removal prior to LC/MS analysis. We recommend guanidinium hydrochloride in place of urea as it does not lead to additional peptide modifications (such as carbamylation) even at elevated temperatures. For the purpose of this chapter, workflow based on RIPA based lysis is presented.
3. We recommend TCEP instead of DTT as reducing agent since it allows for the reduction and alkylation step (with CAA) to be done simultaneously. Additionally, TCEP lacks a strong odor unlike DTT (*see* Chapter 17 for more details).
4. It is important to have peptide mixtures in buffer conditions similar to starting condition of the LC gradient. For example if the gradient starts at pH 10 with buffer A, it is recommended to reconstitute the peptide mixture in Buffer A or add Buffer A to the sample so the pH is similar. This is to allow for proper peptide hydrophobic interaction in similar conditions as the column.
5. Addition of buffers such as ammonium formate is not recommended since they have been shown to cause column degradation in combination with high pH [5, 11]. At 5 mM ammonium hydroxide the pH value is roughly 10.5, due to the absence of any buffer ions the pH value does not drop or increase much in the range of 1–5 mM. Ammonia dissolved up to 25 % will have a concentration of roughly 14–14.5 N. We recommend monitoring pH with pH meter or strips, buffers should be exchanged every few weeks.

6. For HPLC systems with quaternary pumps (systems in which four different lines can be operated simultaneously), we recommend allocating line A to pure water (Milli-Q grade), line B to pure acetonitrile, and line C to higher concentration of ammonium hydroxide (10–50 mM). In this case line C would be running constantly at 10 % of total flow rate to dilute the concentration of the ammonium hydroxide to a final concentration of 1–5 mM. These systems are recommended as line C can be shut off after the fraction collection period and the column washed without ammonium hydroxide.
7. It is important to determine buffer compatibility of columns with manufacturers. We recommend columns with small bead sizes (<5 μM) as they better separate peptides with small peak widths. Columns with smaller internal diameters (<4.6 mm) will require smaller loading amounts of tryptic peptides. In all cases we recommend column lengths of 250 mm since that is sufficient for efficient separation using long gradients. Columns with bigger internal diameters will require the HPLC to handle higher backpressure. We also recommend addition of a guard column with guard cartridges as they can extend the lifespan of the column without affecting separation. The principle of the guard is that the sample is loaded onto the guard column and separated on the main column during the gradient. This prevents larger moieties from affecting the main column and reducing the amount of particulates which may accumulate on the main column. This increases the lifespan of the main separation analytical column since guard cartridges are easily replaceable. This solution is furthermore economically favorable as the cost-benefit of including a replaceable guard column before the separation column can prevent frequent replacement of the analytical column, which is many-fold more expensive than guard column cartridges.
8. It is important that acetonitrile in the sample is removed prior to injection onto the column as it can prevent efficient hydrophobic interactions and broaden peaks as they elute. Typically 30–60 min of drying at 45–60 °C is sufficient to remove acetonitrile. This can also be assessed by eye especially if peptides were eluted off a Sep-Pak in 50 % acetonitrile. The final volume after drying in a speedvac should be half or less of the starting volume since acetonitrile is more volatile compared to water and will dry off faster. It is possible to dry samples for longer periods of time or even to dryness and resuspend in starting buffer with desired volume (we do not however, recommend this since reconstituting peptides can lead to loss of certain peptides and highly acidic buffers are required to resuspend dried peptides, thus making the starting conditions incompatible with high pH, therefore we suggest drying to low volumes but not dryness).

9. For 4.6 mm ID × 250 mm C₁₈ columns flow rates of 1 ml/min are recommended. Typical pressure range when running at 1 ml/min (with low organic) is 150–250 bars depending on bead material. Consult column manufacturer guidelines for further assistance. Instructions for flow rates are usually available from column manufacturer which we highly recommend consulting before operating the HPLC with column. Typically manufactured columns are shipped capped with high organic buffer in the column. It is important that the user recognizes this and washes the column with starting condition of low Buffer B and high Buffer A for several minutes and column volumes. Additionally monitoring the UV and pressure baseline can help determine when the column has reached equilibration as the column has lower backpressure with higher organic solvent. Running the column with several short empty runs with a quick gradient to high organic and back to low organic can help acclimate the column to running buffers for peptide separation. We do not recommend fractionating a peptide mixture on a new column for the first run.
10. Phosphopeptides typically elute earlier in the acetonitrile gradient compared to normal peptides at high pH due to phosphate groups carrying negative charges and preventing hydrophobic interactions with the column [12]. Therefore most of the separation is likely to occur earlier in the gradient (up to 20 % acetonitrile) and thus the gradient length should reflect this when separating phosphopeptides.
11. It is recommended to run BSA peptide mixtures using this method before running any fractions on the column. Advantages of this are to determine the column deterioration and condition since fractionation at high pH may adversely affect the separation efficiency of the column [5, 11]. UV trace of the BSA peptides should be sharp, symmetrical, and the peak width should be small. Several peaks should also be separated across the gradient giving the user an idea of the hydrophobic separation. Under ideal conditions, the UV trace of peptide mixtures should not deviate much from the first few BSA runs and after fractionation of several samples. By having a standard to base the condition of the column, determining column lifespan as well as discarding the column when unfit for proper separations is possible.
12. If the available injection loop of the auto-sampler is small and limits the injection volume other methods of injection of the sample onto the column should be utilized. We recommend injection loops of 500 µl or more. In cases where a larger loop is not present or the final volume is too high, it is possible to inject with an extra line or pump if present. However this runs the risk of contaminating your HPLC system with sample and is not recommended.

13. 96 deep well plates are recommended as they hold 2 ml per well and allow for longer gradient times for collection. Most fraction racks for Eppendorf or other tubes are limited in the number of tubes they may hold. It is recommended to select correct settings for rack type and tubes using the LC control software.
14. It might be necessary to speedvac fractions if performing proteomics analysis in which small amounts can be taken out (i.e., if 2 mg of peptides are fractionated in ten fractions with basic reversed-phase, one can expect 200 μg per fraction, from which only 1 % (2 μg) of the fraction would theoretically be required for proteomics analysis since the injection amounts for nano-LC columns coupled to MS are typically within this range). It is recommended to determine the concentration using Nanodrop A_{280} . Depending on gradient length and number of fractions, the fraction volume can vary from 5 to 15 ml.
15. Monitor wash method with UV and pressure changes, UV absorbance counts should be low relative to UV trace of previous sample. This can be helpful in determining the amount of residual sample if some persists.
16. The higher the injection volume, the longer the loading of the sample onto the nano column takes. For a typical 15 cm nano column (precolumn) on a standard LC (maximum 300 bars), injecting 5 μl of sample at maximum pressure can take from 20 to 30 min. This is reduced with nLC systems which are UPLC (high pressure compatible LCs) which can tolerate up to 1000 bars.

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