INSTRUCTIONS

# Pierce High pH Reversed-Phase Peptide Fractionation Kit

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84868

| Number | Description |
|---|---|
| **84868** | **Pierce High pH Reversed-Phase Peptide Fractionation Kit**, sufficient materials for fractionation of up to 12 proteolytically digested protein samples before LC/MS analysis |

**Kit Contents:**

**Reversed-Phase Fractionation Spin Columns**, 12 columns containing 20mg of resin in a 1:1 water/DMSO slurry

**Triethlyamine (0.1% in water)**, 100mL

**Storage:** Upon receipt store at 4°C. Reagents are shipped at room temperature.

**Note:** Upon receipt, the spin column resin can have a non-uniform appearance, but this will not affect performance. To avoid accidental loss of resin material, do not remove the top screw cap until Step 3 of the Conditioning of the Spin Columns procedure.

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## Introduction

The Thermo Scientific™ Pierce™ High pH Reversed-Phase Peptide Fractionation Kit provides an optimized fractionation protocol and reagents to increase the number of proteins identified from complex samples by liquid chromatography-mass spectrometry (LC-MS) analysis. High-pH reversed-phase chromatography is a robust method of peptide fractionation that separates peptides by hydrophobicity and provides excellent orthogonality to low-pH reversed-phase LC-MS gradients. In contrast to strong cation exchange (SCX) fractionation, high-pH reversed-phase fractions do not require an additional desalting step before LC-MS analysis.

The kit includes a high-pH solution (0.1% triethylamine) and 12 spin columns containing pH-resistant, reversed-phase resin. Each reversed-phase fractionation spin column enables fractionation of 10-100µg of peptide sample using a microcentrifuge. Native, phosphorylated, Thermo Scientific™ Tandem Mass Tag™ (TMT™)-labeled, and other complex peptide mixture samples can be fractionated using the kit. Combining the search results generated by the individual fractions improves protein sequence coverage and increases the number of identified proteins relative to unfractionated samples.

## Procedure Summary

Proteolytic digests of proteins extracted from cells or tissues are loaded onto an equilibrated, high-pH, reversed-phase fractionation spin column. Peptides are bound to the hydrophobic resin under aqueous conditions and desalted by washing the column with water by low-speed centrifugation. A step gradient of increasing acetonitrile concentrations in a volatile high-pH elution solution is then applied to the columns to elute bound peptides into eight different fractions collected by centrifugation. Each fraction is then dried in a vacuum centrifuge (e.g., Thermo Scientific™ SpeedVac™ Vacuum Concentrator) and stored until analysis by mass spectrometry. During LC-MS analysis, peptides in each high-pH fraction are further separated using a low-pH gradient, thus reducing the overall sample complexity and improving the ability to identify low-abundant peptides.

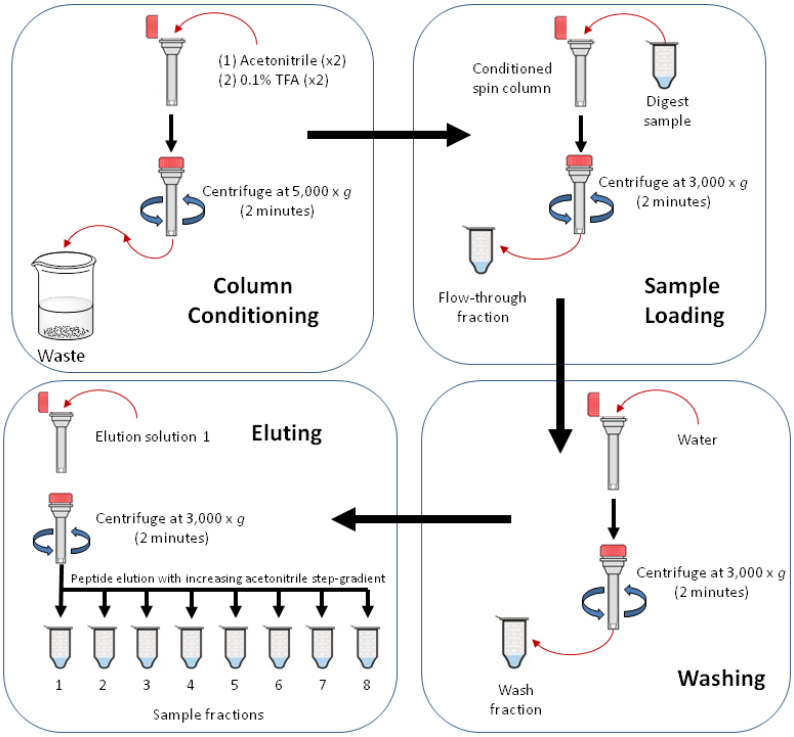


**Figure 1. Spin column conditioning and sample fractionation workflow.**

## Important Product Information

- Do not exceed the recommended centrifugation speeds because this may damage the column frit, causing the resin material to leak, leading to sample loss and/or damage to the LC system.
- Use low protein-binding microcentrifuge tubes to ensure maximum sample recovery.
- Store high-pH buffers in polypropylene tubes at room temperature. Do not store high-pH buffers in glass vessels.
- Avoid sample contamination and direct skin contact with solvents and chemicals. Always wear gloves when handling the spin columns and samples.

## Additional Materials Required

- Trifluoroacetic acid (Product No. 28904)
- Acetonitrile (ACN), LC-MS Grade (Product No. 51101)
- Water, LC-MS Grade (Product No. 51140)
- Thermo Scientific™ Pierce™ Low Protein Binding Microcentrifuge Tubes, 2.0mL (Product No. 88379 or 88380)
- Microcentrifuge with adjustable rotor speed up to 7,000 × *g*
- Vacuum centrifuge

## Material Preparation

- Trifluoroacetic acid (TFA), 0.1% — Prepare 10mL of equilibration solution by adding 10µL of TFA to 10mL of water. Volume is sufficient for equilibration of 12 columns.

- High-pH step-elution solutions — Prepare solutions in 2.0mL tubes according to peptide sample type (Table 1 or Table 2). Different sets of elution solutions are used for unlabeled, native peptides or TMT-labeled samples due to different peptide retention behavior. Note that 300µL of each solution is required per sample. Recommended volumes in Tables 1 and 2 are enough for fractionation of up to three samples.

**Table 1. Preparation of elution solutions for unlabeled, native peptides.**

| Fraction No. | Acetonitrile (%) | Acetonitrile (µL) | Triethylamine (0.1%) (µL) |
|---|---|---|---|
| 1 | 5.0 | 50 | 950 |
| 2 | 7.5 | 75 | 925 |
| 3 | 10.0 | 100 | 900 |
| 4 | 12.5 | 125 | 875 |
| 5 | 15.0 | 150 | 850 |
| 6 | 17.5 | 175 | 825 |
| 7 | 20.0 | 200 | 800 |
| 8 | 50.0 | 500 | 500 |

**Table 2. Preparation of elution solutions for Thermo Scientific TMT-labeled peptides.**

| Fraction No. | Acetonitrile (%) | Acetonitrile (µL) | Triethylamine (0.1%) (µL) |
|---|---|---|---|
| Wash | 5.0 | 50 | 950 |
| 1 | 10.0 | 100 | 900 |
| 2 | 12.5 | 125 | 875 |
| 3 | 15.0 | 150 | 850 |
| 4 | 17.5 | 175 | 825 |
| 5 | 20.0 | 200 | 800 |
| 6 | 22.5 | 225 | 775 |
| 7 | 25.0 | 250 | 750 |
| 8 | 50.0 | 500 | 500 |

## Fractionation of Proteolytic Digests

### A. Conditioning of the Spin Columns

**Note:** Do not exceed recommended centrifugation speeds.

1. Remove the protective white tip from the bottom of the column and discard. Place the column into a 2.0mL sample tube.
2. Centrifuge at 5000 × *g* for 2 minutes to remove the solution and pack the resin material. Discard the liquid.
3. Remove the top screw cap and load 300µL of ACN into the column. Replace the cap, place the spin column back into a 2.0mL sample tube and centrifuge at 5000 × *g* for 2 minutes. Discard ACN and repeat wash step.
4. Wash the spin column twice with 0.1% TFA solution, as described in Step 3. The column is now conditioned and ready for use.

**B. Fractionation of Digest Samples**

**Note:** Each sample requires 300µL of each elution solution. If more than three samples require fractionation, prepare larger volumes of the elution solutions to accommodate all samples.

1. Prepare elution solutions according to Table 1 or Table 2 depending on sample type.
2. Dissolve 10-100µg of digested sample in 300µL of 0.1% TFA solution.

   **Note:** Peptide samples need to be completely dissolved and free of organic solvent (e.g., ACN, DSMO, etc.). If the sample contains urea, make sure that the final concentration of urea is ≤ 1M.
3. Place the spin column into a new 2.0mL sample tube. Load 300µL of the sample solution onto the column, replace the top cap and centrifuge at 3000 × *g* for 2 minutes. Retain eluate as "flow-through" fraction.
4. Place the column into a new 2.0mL sample tube. Load 300µL of water onto the column and centrifuge again to collect the wash. Retain eluate as "wash" fraction.

   Note: TMT-labeled samples require an additional column wash with 300µL of 5% ACN, 0.1% TEA (see Table 2) to remove unreacted TMT reagent.
5. Place the column into a new 2.0mL sample tube. Load 300µL of the appropriate elution solution (e.g., 5% ACN, 0.1% TEA) and centrifuge at 3000 × *g* for 2 minutes to collect the fraction.
6. Repeat Step 5 for the remaining step gradient fractions using the appropriate elution solutions from Table 1 or Table 2 in new 2.0mL sample tubes.
7. Evaporate the liquid contents of each sample tube to dryness using vacuum centrifugation (e.g., SpeedVac concentrator).
8. Re-suspend dry samples in an appropriate volume of 0.1% formic acid (FA) before LC-MS analysis.
9. **Optional:** Determine the peptide concentration and yield with a peptide quantitation assay, so equivalent sample amounts can be analyzed by LC-MS.

## Troubleshooting

| Problem | Possible Cause | Solution |
|---|---|---|
| Low peptide yields | Low protein yield following lysis and protein extraction procedure | Estimate protein concentration using BCA assay |
| | | Use an alternative protein extraction procedure |
| | Lyophilized/dried peptide samples were not completely solubilized before sample loading onto the spin column | Increase vortexing/sonication time to completely dissolve the dried peptide sample |
| Unsuccessful fractionation | Incorrect centrifuge speeds used for fractionation | Ensure proper centrifuge speed is used [in (× *g*)]. To convert from revolutions per minute (rpm) to *g*, use the following formula: $g = (1.118 \times 10^{-5})\ RS^2$ where *g* is the relative centrifugal force, R is the rotor radius in centimeters, and S is the centrifuge speed in rpm. For example, centrifugation of a sample at 5,000 rpm in a microcentrifuge having a rotor radius of 7cm will deliver a centrifugal force of 1,957 × *g* |
| Low peptide/protein identification numbers | Low sample load (< 10µg) | Estimate peptide concentration using the Thermo Scientific™ Pierce™ Quantitative Fluorometric Peptide Assay (Product No. 23290) or Thermo Scientific™ Pierce™ Quantitative Colorimetric Peptide Assay (Product No. 23275) |
| | | Use low protein-binding tubes for handling of the samples and fraction collection |
| | Incorrect chromatography or mass spectrometer instrument settings | Consult instrument user manuals or online resources to determine the optimal instrument settings for your system |
| | | Verify LC-MS system performance with the Thermo Scientific™ Pierce™ HeLa Digest Protein Standard (Product No. 88328) |

## Related Thermo Scientific Products

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| **90061** | **TMTsixplex™ Isobaric Label Reagent Set,** 1 × 0.8mg |
| **90064** | **TMTsixplex Isobaric Mass Tagging Kit** |
| **90110** | **TMT10plex™ Isobaric Label Reagent Set,** 1 × 0.8mg |
| **90113** | **TMT10plex Isobaric Mass Tag Labeling Kit,** 30 rxns |
| **90101** | **iodoTMTsixplex™ Label Reagent Set,** 1 × 0.2mg |
| **90103** | **iodoTMTsixplex Isobaric Mass Tag Labeling Kit** |
| **90401** | **aminoxyTMTsixplex™ Label Reagent Set,** 1 × 0.2mg |
| **84840** | **Pierce™ Mass Spec Sample Prep Kit for Cultured Cells** |
| **88328** | **Pierce HeLa Digest Protein Standard** |
| **23227** | **BCA Protein Assay Kit** |
| **23275** | **Pierce Quantitative Colorimetric Peptide Assay** |
| **23290** | **Pierce Quantitative Fluorometric Peptide Assay** |
| **90057** | **Pierce™ Trypsin Protease, MS Grade** |
| **28904** | **Trifluoroacetic Acid, Sequanal Grade** |
| **51140** | **Water, LC-MS Grade** |
| **51101** | **Acetonitrile (ACN), LC-MS Grade** |

### General References


Feng Yang, Yufeng Shen, David G. Camp II, Richard D. Smith (2012). High pH reversed-phase chromatography with fraction concatenations as an alternative to strong-cation exchange chromatography for two-dimensional proteomic analysis. *Expert Rev Proteomics* **9(2)** 129-34.

Mohammad Pirmoradian, Harshavardhan Budamgunta, Konstantin Chingin, Bo Zhang, Juan Astorga-Wells, Roman A. Zubarev (2013). Rapid and deep human proteome analysis by single-dimension shotgun proteomics. *Mol Cell Proteomics* **12(11)** 3330-8.

Washburn, M.P.; Wolter, D.; Yates, J.R. 3rd (2001). Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat Biotechnol* **19(3)** 242-7.





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