INSTRUCTIONS

Pierce TiO_2 Phosphopeptide Enrichment and Clean-up Kit

88301 88303 2169.7

Number	Description
88301	**Pierce TiO_2 Phosphopeptide Enrichment and Clean-up Kit** **Kit Contents:** **TiO_2 Spin Tips,** 24 each **Trifluoroacetic Acid** (TFA), 1mL **Lactic Acid (90%),** 2mL **Pyrrolidine,** 200µL **Centrifuge Column Adaptors,** 6 each **Pierce Graphite Spin Columns,** 24 columns
88303	**Pierce TiO_2 Phosphopeptide Enrichment Spin Tips,** 96 tips and 24 adapters

NOTE: Trifluoroacetic Acid, Lactic Acid and Pyrrolidine components are shipped separately from the remainder of the kit.

Storage: Upon receipt store at 4°C. Product shipped at ambient temperature.

Introduction

The Thermo Scientific™ Pierce™ TiO_2 Phosphopeptide Enrichment and Clean-up Kit enables efficient isolation of phosphorylated peptides from complex and fractionated protein digests for analysis by mass spectrometry (MS). Spherical porous titanium dioxide (TiO_2) combined with optimized buffers provide enhanced enrichment and identification of phosphopeptides with minimal nonspecific binding. The spin-column format is fast and easy to use and can enrich up to 100µg of phosphopeptides from 300-1000µg of digested protein sample. The kit's optimized protocol, buffer components and graphite spin columns result in a high yield of clean phosphopeptide samples ready for MS analysis.

Phosphorylation is a protein modification essential to biological functions such as cell signaling, growth, differentiation and division, and programmed cell death; however, phosphopeptides have high hydrophilicity and are low in abundance, resulting in poor chromatography, ionization and fragmentation. Phosphopeptide enrichment is therefore essential to successful MS analysis. The Pierce TiO_2 Phosphopeptide Enrichment and Clean-up Kit is compatible with our lysis, reduction, alkylation, digestion and graphite spin columns to provide a complete workflow for phosphopeptide enrichment and identification.

Important Product Information

- For best results perform all experiments using a centrifuge. Using a pipette to draw and expel liquid through the spin tip is strongly discouraged and may result in poor, varied results. Note that the Centrifuge Column Adaptors are reusable.
- For optimal results, desalt peptide samples with C18 before phosphopeptide enrichment.[1]
- Because of the viscosity and ionic components in the binding and wash buffers for the TiO_2 tips and the hydrophilicity of enriched phosphopeptides, performing the graphite clean-up is necessary before MS analysis. Make sure samples are free of detergents before desalting with C18 or graphite cartridges.
- For optimal results, proceed with the entire procedure in a timely manner and avoid excessive resin drying between steps.
- Plastics used during handling of peptide samples can introduce contaminants that interfere with MS analysis and result in sample loss from nonspecific adsorption. Use high-quality receiver tubes. If necessary, receiver tubes used for final collection may be rinsed with 70% ACN/0.1% TFA. Minimizing sample transfers and freeze-thaw cycles before analysis will help minimize plastic contamination and sample loss.

Procedure for Phosphopeptide Enrichment

A. Additional Materials Required

- Ammonium hydroxide
- Acetonitrile
- Ultrapure water

B. Material Preparation

Note: Prepare buffer reagents just before performing experiments.

Buffer	Component	1-6 Samples	24 Samples
Buffer A	Ultrapure Water	100µL	402µL
	Acetonitrile	401µL	1,601µL
	TFA (100%)	2.0µL	8µL
	Total	**503µL**	**2,011µL**
Buffer B*	Buffer A	1mL	5mL
	Lactic Acid (90%)[§]	400µL	2mL
	Total	**1.4mL**	**7mL**
Elution Buffer 1	Ammonium Hydroxide (30%)[†]	20µL	100µL
	Ultrapure Water	380µL	1.9mL
	Total	**400µL**	**2mL**
Elution Buffer 2*	Pyrrolidine	20µL	200µL
	Ultrapure Water	380µL	3.8mL
	Total	**400µL**	**4mL**
2.5% TFA	Ultrapure Water	980µL	2.9mL
	TFA (100%)	25µL	75µL
	Total	**1mL**	**3mL**

*If reagents will be consumed within one week of dissolution, Buffer B and Elution Buffer 2 may be prepared by diluting the 90% lactic acid and pyrrolidine, respectively, in the provided bottles.
[§]Lactic acid is viscous. Aspirate slowly or use a wide-bore tip.
[†]Ammonium hydroxide solutions are generally supplied at ~30% (w/v).

C. Column Preparation

1. Place a Centrifuge Column Adaptor in a collection tube and insert a TiO_2 Spin Tip into the adaptor. Note that the Centrifuge Column Adaptors are reusable.
2. Add 20µL of Buffer A and centrifuge at 3000 × *g* for 2 minutes. Discard the flow-through.
3. Add 20µL of Buffer B and centrifuge at 3000 × *g* for 2 minutes. Discard the flow-through.

D. Phosphopeptide Binding

1. Suspend peptide sample in 150µL of Buffer B and apply to the spin tip. Centrifuge at 1000 × *g* for 10 minutes.
2. Reapply sample to the spin tip and centrifuge at 1000 × *g* for 10 minutes. If desired, retain the flow-through for analysis.
3. Wash column by adding 20µL of Buffer B. Centrifuge at 3000 × *g* for 2 minutes.
4. Wash column by adding 20µL of Buffer A. Centrifuge at 3000 × *g* for 2 minutes. Repeat this wash step twice.

E. Elution

1. Place the spin tip in new collection tube and add 50µL of Elution Buffer 1. Centrifuge at 1000 × *g* for 5 minutes.
2. Using same collection tube, add 50µL of Elution Buffer 2 to the spin tip. Centrifuge at 1000 × *g* for 5 minutes.
3. Acidify the elution fraction by adding 100µL of 2.5% TFA. Ensure the sample pH is 2.0-2.5 for proper binding and desalting on C18 or graphite resins.

   Note: Process the eluted sample with the Pierce Graphite Spin Columns before MS analysis.

Procedure for Graphite Clean-up of Phosphopeptides

A. Additional Materials Required

Note: TFA solutions can be prepared from the 100% TFA provided with the kit.

- 100% Acetonitrile (100µL per sample)
- 1.0% TFA (600µL per sample)
- 0.1% TFA (20µL per sample)
- 1M NH_4OH (200µL per sample)
- 0.1% Formic acid in 50% acetonitrile (400µL per sample)
- 1.5mL Microcentrifuge tubes

B. Column Preparation

1. Remove top and bottom cap from a graphite spin column. Place column into a 1.5mL tube and centrifuge at 2000 × *g* for 1 minute to remove the storage buffer.
2. Add 100µL of 1M NH_4OH and centrifuge at 2000 × *g* for 1 minute. Discard the flow-through. Repeat this step once.
3. Activate graphite by adding 100µL of acetonitrile. Centrifuge at 2000 × *g* for 1 minute and discard the flow-through.
4. Add 100µL of 1% TFA and centrifuge at 2000 × *g* for 1 minute. Discard the flow-through. Repeat this step once.

C. Sample Binding and Elution

1. Place column into a new collection tube and apply sample on top of the resin bed. Allow binding for 10 minutes with periodic vortex mixing.
2. Centrifuge at 1000 × *g* for 3 minutes. Discard the flow-through.
3. Place column into a new collection tube. Wash column by adding 200µL of 1.0% TFA and centrifuging at 2000 × *g* for 1 minute. Discard the flow-through. Repeat this step once.
4. Place column into new collection tube. Add 100µL of 0.1% formic acid in 50% acetonitrile to elute sample. Centrifuge at 2000 × *g* for 1 minute. Repeat this step three more times using the same collection tube for a total elution volume of 400µL.

   Optional: To reduce final volume, elute with 100µL of 0.1% formic acid in 50% acetonitrile. Re-apply eluate three more times for a total elution volume of 100µL.
5. Gently dry sample in a vacuum evaporator. For MALDI-MS analysis, carefully suspend sample in 1-2µL of matrix solution prepared just before use. For LC-ESI applications, suspend sample in 20µL of 0.1% TFA or an appropriate buffer.

Troubleshooting for Phosphopeptide Enrichment

Problem	Possible Cause	Solution
Nonspecific peptide binding	Sample or Buffer B was at pH > 3.5	Make sure pH is < 3.5 by increasing the TFA concentration
	Wash volume was insufficient for the peptide concentration	Increase the number or volume of washes
	Nonspecific peptide is highly acidic	Increase TFA concentration or add 100mM NaCl to Buffer B or increase the number of washes
	Nonspecific peptide is highly hydrophobic	Increase amount of acetonitrile in Buffer B to 80%
No phosphopeptide recovered	Phosphopeptide concentration was too low	Increase amount of sample added or reduce elution buffer volume
	Poor MS sample quality	Desalt enriched samples before MS analysis
	Sample was overly buffered and pH remains > 5.5	Lower pH to < 3.0 by adding TFA
	High levels of interfering agents such as EDTA, free metals or ammonium bicarbonate were in the sample	Desalt enriched samples before MS analysis

Troubleshooting for Phosphopeptide Clean-Up

Problem	Possible Cause	Solution
Poor or incomplete sample binding	High pH and lack of ion-pairing agents	Add TFA to sample before binding
	Lack of mixing during binding period	Ensure that graphite is well suspended in sample during binding
Poor or incomplete sample recovery	Peptides bound to plastics, which caused significant peptide loss	Minimize contact with plastics, excessive drying and storage at low concentration
	Detection limits of the specific application	Ensure sample is within the detection limit of the application – limits vary considerably based on the application and instrumentation
Graphite material is present in the flow-through or washes	A detergent or detergent-like compound was in the sample	Remove detergent from the protein or peptide sample with detergent-removal columns (Product No. 87777) before desalting or remove detergent from protein sample by acetone precipitation before digestion

Related Thermo Scientific Products

88300 **Pierce Fe-NTA Phosphopeptide Enrichment Kit**
90003 **Pierce Phosphoprotein Enrichment Kit**
88811 **Pierce Magnetic Titanium Dioxide Phosphopeptide Enrichment Kit**
87777 **Pierce Detergent Removal Spin Column, 0.5mL,** 25 columns
88302 **Pierce Graphite Spin Columns,** 30 columns
87782 **Pierce C18 Tips, 10µL,** 96 tips
87784 **Pierce C18 Tips, 100µL,** 96 tips
89870 **Pierce C18 Spin Columns**, 25 columns

Cited Reference

1. Villén, J. and Gygi, S.P. (2008). The SCX/IMAC enrichment approach for global phosphorylation analysis by mass spectrometry. *Nat Protoc* **3(10)**:1630-8.

General References

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Kyono, Y., *et al.* (2008). Successive and selective release of phosphorylated peptides captured by hydroxy acid-modified metal oxide chromatography. *J Proteome Res* **7(10):**4585-93.

Carrascal, M., *et al*. (2008). Phosphorylation analysis of primary human T lymphocytes using sequential IMAC and titanium oxide enrichment. *J Proteome Res* **7(12):**5167-76.

Larsen, M.R., *et al.* (2004). Improved detection of hydrophilic phosphopeptides using graphite powder microcolumns and mass spectrometry: evidence for *in vivo* doubly phosphorylated dynamin I and dynamin III. *Mol Cell Proteomics* **3:**456-65.

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