Titansphere Phos-TiO Tip Instruction Manual

1 Introduction

Thank you very much for purchasing this product.
To maintain optimum performance, read the following instructions before use.

2 Safety Precautions

- Store and handle Phos-TiO spin tips carefully, as dropping or powerfully tapping or “flicking” the tips can disrupt the uniformly packed sorbent bed, which results in poor- or no recovery of sample as sample can then move through the tip without being forced to interact with the sorbent surface.
- Autoclave can not be applicable.
- Please store the product in a low humidity environment such as a desiccator after opening the original packaging.
- The Spin Tip is a disposable product and not for reuse.
- The maximum centrifugal acceleration is 10,000 xg.
- The maximum operating temperature is 40℃.
- Use highly pure acetonitrile for a mass spectrometer (MS) analysis.

3 Product Features

The Titansphere Phos-TiO Tip is sample preparation tip packed with Titansphere synthetic spherically porous titanium dioxide (TiO_2), suited for purifying and enriching phosphopeptide in research areas. This tip column is a customized product from Titansphere TiO for high performance liquid chromatography (HPLC) to separate or purify phosphopeptide.

Our Titansphere Phos-TiO Tip can be used for the purification and enrichment of phosphopeptide directly from cell culture medium, and/or samples roughly purified by immuno-precipitation, SDS-PAGE or two-dimensional electrophoresis.

4 Unpacking

The contents of each type of Phos-TiO Tip are detailed below. Upon receipt of a product, please verify that the proper quantity of each component is present and not damaged (cracked or crushed).

Titansphere sorbent mass/ Tip Volume	24 pcs (6 x 4 pack)	96 pcs (6 x 16 pack)
	Cat. No.	Cat. No.
1 mg / 10 µL	5010-21302	5010-21303
1 mg / 200 µL	5010-21316	5010-21317
3 mg / 200 µL	5010-21307	5010-21308

5 How to use

5-1 Example of Protocol (1)
***Use 1 mg/10 µL or 3 mg/ 200 µL Tip**

■Reagent Preparation
- High-purity acetonitrile
- High-purity trifluoroacetic acid (TFA)
- DL-Lactic acid (Recommend: Fujifilm Wako Chemicals 128-00056, Sigma-Aldrich SAJ first grade 18-0050, JIS special grade 18-0040)
- Ammonium Hydroxide solution
- Pyrrolidine

- How to make Buffer A:
  Prepare 2% TFA solution. Use 2% TFA solution and Acetonitrile to make Buffer A.

- How to make Buffer B:
  Use Lactic acid and Buffer A to make Buffer B.

Lactic acid is used as an inhibitor of non-specific peptides adsorbing from crude sample such as a cell lysate to titanium dioxide.

Buffer A	2% TFA solution	1 mL
	Acetonitrile	4 mL
	Total	5 mL(Use 3 mL for making Buffer B)

Buffer B	Lactic acid	1 mL
	Buffer A	3 mL
	Total	4 mL

- Prepare 5% Pyrrolidine solution
- Prepare 5% Ammonium Hydroxide solution

5% ammonium aqueous solution tends to elute a hydrophilic phosphopeptides and 5% pyrrolidine aqueous solution tends to elute a hydrophobic phosphopeptides.

Note : Use the reagent made on the day of the experiment.
Prepare the reagent just before performing the experiment.
Lactic acid has a high viscosity. Aspirate slowly.

- Use protein digest (sample peptides) after finishing the reduction and alkylation procedure.
- Make sure that all applied solutions, such as conditioning, adsorption/sample (load), rinsing/washing, solutions are completely eluted from the Spin Tip after each step in the centrifuge procedure.
- With the exception of the adsorption step, as indicated below, use the same amount of Buffer volumes for both the 10 µL and 200 µL spin tips.

*RT:Room temperature

Spin Tip
- Connect a centrifugal adaptor to a Waste Fluid Tube, and insert the Spin Tip into tube. (Figure. 1).

Conditioning
- Add 20 µL of Buffer A then centrifuge (3,000 xg, 2 min, RT).

Equilibration
- Add 20 µL of Buffer B then centrifuge (3,000 xg, 2 min, RT)
- Remove the 40 µL of waste fluid from a tube

Adsorption
- Add 15～50 µL of the sample and 100 µL Buffer B into the Spin Tip.
  ＊In the case of 10 µL tip, add 15 µL of the sample and 50 µL of Buffer B into the Spin Tip.
- To mix the sample with Buffer B, repeat pipetting three times within the Spin Tip. (Figure. 2)
- Centrifuge (1,000 xg, 10 min, RT)
  ＊High centrifugal speed may cause to decrease the recovery rate.
- Put the sample in the tube back into the Spin Tip again and centrifuge (1,000 xg, 10 min, RT)
- Remove the sample and Buffer B collected in the tube.

Rinsing
- Add 20 µL of Buffer B and centrifuge (3,000 xg, 2 min, RT)
- Add 20 µL of Buffer A and centrifuge (3,000 xg, 2 min, RT)
  ＊Add of Buffer A, repeat twice.

Elution
- Put the Spin Tip into the recovery tube.
- Add 50 µL of 5% ammonium hydroxide solution and centrifuge. (1,000 xg, 5 min, RT)
- Add 50 µL of 5% pyrrolidine solution and centrifuge. (1,000 xg, 5 min, RT)

Phosphopeptide

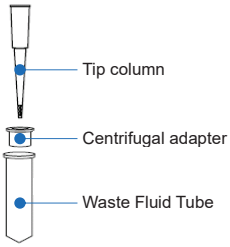

Figure 1. Setting the spin tip

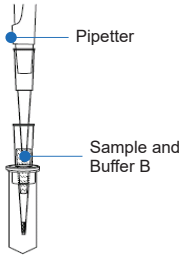

Figure 2. Method of mixing sample and Buffer B

■ Cleanup and desalting

＊＊Example protocol for desalting (Ⅰ)＊＊

To remove Lactic acid from enriched phosphopeptides using a reversed solid phase extraction such as GL-Tip SDB (Cat. No.7820-11200), GL-Tip GC (Cat. No.7820-11201).

pH adjustment and preparation
- Collected/purified sample 100 µL (500 mM disodium hydrogen phosphate) and 100 µL (5% Ammonia solution 50 µL, 5% Pyrrolidine solution 50 µL) from Titansphere Phos-TiO Tip.
  Add either 100 µL of 20% phosphoric acid solution or 20% TFA solution to the above collected/purified sample 100 µL.
  Mix the solutions and confirm with a litmus paper if the pH of solution is acidic.
- Preparation of Buffers:
  Buffer X (0.1% TFA in Water:CH_3CN＝95:5)
  Buffer Y (0.1% TFA in Water:CH_3CN＝20:80)

Desalting and clean up

Use “GL-Tip SDB” and/or “GL-Tip GC”
＊”GL-Tip GC” is recommended to recover hydrophilic peptides that cannot be retained by GL-Tip SDB. Details are shown below.

- Conditioning and equilibration:
  Add 20 µL of Buffer Y, then centrifuge. (3,000 xg, 2 min, RT)
  Add 20 µL of Buffer X, then centrifuge. (3,000 xg, 2 min, RT)
- Adsorption: Add sample then centrifuge. (3,000 xg, 5 min, RT)
  (Flow through eluate in the tube after centrifuge can be desalted by GL-Tip GC. Use a new tube before adsorption with GL-Tip GC.)

Flow Through Eluate → GL-Tip GC Operation Manual
This Protocol is same as GL-Tip SDB.
- [1] Conditioning and equilibration:
  Add 20 µL of Buffer Y, then centrifuge. (3,000 xg, 2 min, RT)
  Add 20 µL of Buffer X, then centrifuge. (3,000 xg, 2 min, RT)
- [2] Adsorption:
  Add flow through eluate then centrifuge. (3,000 xg, 5 min, RT)
- [3] Rinsing:
  Add 20 µL of Buffer X, then centrifuge. (3,000 xg, 2 min, RT)
- [4] Elution:
  Add 50 µL of Buffer Y, then centrifuge. (3,000 xg, 3 min, RT)

Mix this eluate with the eluate obtained by GL-Tip SDB.

- Rinsing:
  Add 20 µL of Buffer X and centrifuge. (3,000 xg, 2 min, RT)
- Elution:
  Add 50 µL of Buffer Y and centrifuge. (3,000 xg, 3 min, RT)
- Centrifuge and evaporate the admixed eluate for 20 min.
- Dissolve it with a solution such as 0.1% TFA, 5% CH_3CN etc.

MS

5-2 Example of Protocol (2)
***Use 1 mg/200 µL or 3 mg/ 200 µL Tip**

For purification and enrichment of multiply phosphopeptide by fractionation of singly and multiply phosphopeptides on elute process.

■Reagent Preparation
- High-purity acetonitrile
- High-purity trifluoroacetic acid (TFA)
- DL-Lactic acid (Recommend: Fujifilm Wako Chemicals 128-00056, Sigma-Aldrich SAJ first grade 18-0050, JIS special grade 18-0040)
- Methylphosphonic acid (Sigma-Aldrich 289868)
- Disodium hydrogen phosphate
- Ammonium Hydroxide solution
- Pyrrolidine

- How to make Buffer A:
  Prepare 2% TFA solution. Use 2% TFA solution and Acetonitrile to make Buffer A.

- How to make Buffer B:
  Use Lactic acid and Buffer A to make Buffer B.

- How to make Buffer C to selectively elute singly phosphopeptides:
  Prepare 20 mM Methylphosphonic acid (adjusted to pH2.0 with NaOH) / 20% acetonitrile solution.

Buffer A	2% TFA solution	1 mL
	Acetonitrile	4 mL
	Total	5 mL(Use 3 mL for making Buffer B)

Buffer B	Lactic acid	1 mL
	Buffer A	3 mL
	Total	4 mL

- Prepare 500 mM disodium hydrogen phosphate solution (no pH adjustment).
- Prepare 5% Pyrrolidine solution.
- Prepare 5% Ammonium Hydroxide solution.

Note : Use the reagent made on the day of the experiment.
Prepare the reagent just before performing the experiment. Lactic acid has a high viscosity. Aspirate slowly.

- Use protein digest (sample peptides) after finishing the reduction and alkylation procedure.
- Make sure that all applied solutions, such as conditioning, adsorption/sample (load), rinsing/washing, solutions are completely eluted from the Spin Tip after each step in the centrifuge procedure.

∗RT:Room temperature

Spin Tip

Connect a centrifugal adaptor to a Waste Fluid Tube, and insert the Spin Tip into tube. (Figure. 1)

Conditioning

Add 20 µL of Buffer A then centrifuge (3,000 xg, 2 min, RT)

Equilibration

Add 40 µL of Buffer B then centrifuge (3,000 xg, 2 min, RT)
Remove the 60 µL of waste fluid from a tube

Adsorption

Add 25～100 µL of the sample and an equal amount of Buffer B into the Spin Tip.
To mix the sample with Buffer B, repeat pipetting three times within the Spin Tip. (Figure. 2)
Centrifuge (1,000 xg, 10 min, RT)
∗ High centrifugal speed may cause to decrease the recovery rate.
Put the sample in the tube back into the Spin Tip again and centrifuge (1,000 xg, 10 min, RT)
Remove the sample and Buffer B collected in the tube.

Rinsing

Add 20 µL of Buffer B and centrifuge (3,000 xg, 2 min, RT)
Add 20 µL of Buffer A and centrifuge (3,000 xg, 2 min, RT)
∗ Add of Buffer A, repeat twice.

Elution

Put the Spin Tip into the recovery tube.
Add 250 µL of Buffer C and centrifuge. (3,000 *xg*. 2 min, RT)
(1mg Tip : Add of buffer C Repeat twice, 3 mg Tip: four times)
Singly phosphopeptides fraction
Put the Spin Tip into the new recovery tube
Add 100 µL of 500 mM disodium hydrogen phosphate and centrifuge.
(1,000 *xg*. 5 min, RT)
Put the Spin Tip into the new recovery tube
Add 50 µL of 5% ammonium hydroxide solution and centrifuge.
(1,000 *xg*. 5 min, RT)
Add 50 µL of 5% pyrrolidine solution and centrifuge.
(1,000 *xg*. 5 min, RT)
Multiply phosphopeptides fraction

Phosphopeptide

■ Cleanup and desalting
The desalting operation of 500 mM disodium hydrogen phosphonate, 5% ammonia solution and 5% pyrrolidine solution containing much multiply phosphopeptides refer to "Example protocol for desalting(I). If you desalt Buffer C containing much singly phosphopeptides, please refer to following "Example protocol for desalting (II).

＊＊Example protocol for desalting (Ⅱ)＊＊
Buffer C eluted from spin tip is diluted five times with a 20% phosphoric acid solution or 20% TFA solution to decrease the concentration of acetonitrile. Because sample volume increase more, recommend following desalting columns.

Titansphere sorbent mass	Buffer C (Volume)	After five times dilution (Volume)	Recommend desalting column (Column volume)	Sample capacity	Minimum elution volume
1 mg Tip	500 µL	2.5 mL	MonoSpin C18 (800 µL)	100 µg	50 µL
3 mg Tip	1 mL	5 mL	MonoSpin L C18 (8 mL)	1 mg	500 µL

Note: In case of 1 mg Tip using MonoSpin C18,
sample have to load to desalting column 3~4 times.
MonoSpin L C18 can be treated 2.5 mL volume at one time.

Desalting and clean up

Use MonoSpin L C18 (Cat. No.7510-11320) or
MonoSpin C18 (Cat. No.5010-21700)
*Note: (volume) indicates MonoSpin C18
Preparation of Buffers:
Buffer X : 0.1% TFA in Water:CH_3CN＝95:5
Buffer Y : 0.1% TFA in Water:CH_3CN＝20:80
Conditioning and equilibration:
Add 500 µL (50 µL) of Buffer Y, then centrifuge. (3,000 xg, 2 min, RT)
Add 500 µL (50 µL) of Buffer X, then centrifuge. (3,000 xg, 2 min, RT)
Adsorption:
Add sample then centrifuge. (3,000 xg, 5 min, RT)
Rinsing:
Add 1 mL (100 µL) of Buffer X and centrifuge. (3,000 xg, 2 min, RT)
Elution:
Add 500 µL (50 µL) of Buffer Y and centrifuge. (3,000 xg, 3 min, RT)
Evaporate the eluate and dissolve with a solution such as 0.1% TFA, 5% CH_3CN.

MS

6 References and FAQ

- Phosphopeptide enrichment by aliphatic hydroxy acid-modified metal oxide chromatography for nano-LC-MS/MS in proteomics applications Sugiyama N.et.al. Mol Cell Proteomics, 6, 1103-1109, 2007
- Successive and Selective Release of Phosphorylated Peptides Captured by Hydroxy Acid-Modified Metal Oxide Chromatography. Kyono Y. et.al. J Proteome Res.,7, 4585-93, 2008
- Extended coverage of mono- and multi-phosphorylated peptides from a single titanium dioxide microcolumn, Wakabayashi M. et al. Ana Chem., 87, 10213-21, 2015

Troubles	Possible Causes	Countermeasure
No phosphopeptide Recovery	Low peptide concentration	Increase peptide concentration to 1 mg/mL.
Many interfering peaks in addition to phosphopeptide are detected	Insufficient rinsing	Increase the elution volume of Buffer B. Increase the acetonitrile concentration of Buffer B for rinsing.
	Insufficient rinsing	Increase the number of rinsings with Buffer B.
Poor recovery rate	Low adsorption	Increase the number of adsorption procedure.
	Solution from the previous step is incomplete	Confirm the liquid is completely eluted after the centrifugal procedure and increase duration if needed.
	The tip end touches or becomes submerged in the Waste/eluted fluid	The recovery rate and selectivity deteriorates if the tip end touches the waste fluid. Particularly when the sample volume is large, make sure that the waste fluid is completely removed after adsorption so a gap remains between tip and waste solutions.
Spectrum such as m/z = 486, 630, 774 appears	Insufficient rinsing	Use Buffer A and rinse several times.
Low fractionation rate *Example of protocol (2)	Strong adsorption of singly phosphopeptides	Increase twice volume of Buffer B to sample at adsorption process.
	Insufficient elution	Increase the number of elution with Buffer C.

7 Storage

- Store the Titansphere Phos-TiO spin tip in a clean and dark place with a constant temperature
- Spin tip is 6 pcs in 1 package. After opening, recommend that use up all 6 pcs. In case of storage, keep the unpacked tips in a low humidity environment such as a desiccator.

Titansphere Phos-TiO Tip is manufactured, inspected, packed and shipped under our strict standards of quality control. Please contact us if you find any problems with the performance of the product.
This product is intended for research only. We do no warranty this product for any particular purpose other than research of phosphopeptides and as described in this instruction.

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