

Pierce[®] Magnetic Titanium Dioxide Phosphopeptide Enrichment Kit

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Number	Description
88811	<p>Pierce Magnetic Titanium Dioxide Phosphopeptide Enrichment Kit, contains sufficient reagents for enriching phosphopeptides from 96 complex biological samples</p> <p>Kit Contents:</p> <p>TiO₂ Magnetic Beads, 20X, 1 ml</p> <p>Binding Buffer, 100 ml</p> <p>Washing Buffer, 25 ml</p> <p>Elution Buffer, 3 ml</p> <p>Thermo-Fast 96 Robotic PCR Plate, 0.2 ml well volume, 2 plates</p>
88812	<p>Pierce Magnetic Titanium Dioxide Phosphopeptide Enrichment Kit-Trial Kit, contains sufficient reagents for enriching phosphopeptides from 24 complex biological samples</p> <p>Kit Contents:</p> <p>TiO₂ Magnetic Beads, 20X, 0.25 ml</p> <p>Binding Buffer, 100 ml</p> <p>Washing Buffer, 25 ml</p> <p>Elution Buffer, 3 ml</p> <p>Thermo-Fast 96 Robotic PCR Plate, 0.2 ml well volume, 2 plates</p> <p>Storage: Upon receipt store at 4°C. Product shipped on an ice pack.</p>

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Introduction

Phosphorylation events in complex biological samples are transitory and typically present in low abundance, making them difficult to detect. The Thermo Scientific Pierce Magnetic Titanium Dioxide Phosphopeptide Enrichment Kit uses titanium dioxide-coated magnetic beads to selectively enrich phosphopeptides from complex mixtures such as digested cell lysates. The buffers and assay protocols included are optimized for efficient capture in less than 30 minutes with minimal binding of non-phosphorylated peptides. Purification is achieved either manually using a magnetic stand or by automation using a KingFisher Flex or KingFisher 96 Instrument.

Cell signaling networks are invariably dependent upon protein phosphorylation, a reversible post-translational modification, for regulation. Approximately 30% of proteins comprising the eukaryotic proteome are likely to undergo some form of phosphorylation event. Understanding the state of signaling pathways and protein phosphorylation cascades under a variety

of conditions is necessary for studying diseases such as cancer. When paired with appropriate sample processing, the identification and quantification of phosphopeptides by mass spectrometry (MS) is a powerful method for studying disease and identifying biomarkers. Samples isolated with this phosphopeptide enrichment kit are ready for direct analysis on a wide variety of MS platforms including the Thermo Scientific LTQ Orbitrap, LTQ and LTQ FT Mass Spectrometers.

Important Product Information

- A wide variety of methods are used to generate phosphopeptide samples. Optimization of sample stimulation, protein isolation and protein digestion procedures are application-specific; however, for best results, use a phosphatase inhibitor, such as the Thermo Scientific Halt Phosphatase Inhibitor Cocktail (Product No. 78420), throughout the isolation process to prevent rapid loss of phosphorylation events.
- Before processing, protein samples need to be reduced, alkylated and digested into peptide fragments. For digestion, use highly specific proteases such as Thermo Scientific MS-grade Trypsin (Product No. 90055) or Lys-C (Product No. 90051).
- For efficient sample handling during manual processing, use the 96-well PCR plates provided with the kit and a 96-well magnetic plate separator.
- Do not freeze or allow prolonged drying of TiO₂ Magnetic Beads. Freezing or excessive drying can cause the beads to lose binding activity.
- For analysis by LC-MS/MS, lyophilize the samples as a final step after processing the beads, and reconstitute them in 5% acetonitrile/0.1% formic acid in water.
- The sample fractions not bound to the beads can be analyzed by mass spectrometry; however, these samples require additional clean-up, such as drying and C18 desalting.
- Avoid phosphate-based buffers during sample preparation because the phosphate anions might interfere with phosphopeptide enrichment.
- Peptides containing acidic residues might reduce phosphopeptide enrichment because the carboxyl groups can interact with the TiO₂ surface. Methylation of the carboxyl groups before phosphopeptide enrichment can improve recovery of phosphopeptides. See the Additional Information Section for a methylation procedure.
- Concentrate and desalt the sample before phosphopeptide enrichment (see the Additional Information Section).

Procedure for Manual Enrichment of Phosphopeptides

A. Additional Materials Required (see the Related Products Section for ordering information)

- 96-well magnetic plate separator
- Sample peptide mixture
- 50 ml polypropylene conical centrifuge tubes
- 15 ml polypropylene conical centrifuge tubes
- Acetonitrile
- Formic acid
- Vacuum concentrator with a 96-well plate rotor

B. Prepare the Binding and Washing Buffers

Note: Prepare buffers just before each experiment.

Binding Buffer	Multiply the number of samples by 1,590 µl to determine the total volume of buffer required. Add 5% for excess. Combine equal volumes of acetonitrile and Binding Buffer in a 50 ml polypropylene conical tube and mix to obtain a homogeneous solution. Close the tube tightly to prevent acetonitrile evaporation.
Washing Buffer	Multiply the number of samples by 200 µl to determine the total volume of buffer required. Add 5% for excess. Combine equal volumes of acetonitrile and Washing Buffer in a 15 ml polypropylene conical tube and mix to obtain a homogeneous solution. Close the tube tightly to prevent acetonitrile evaporation.

C. Prepare the TiO₂ Magnetic Beads

- Use a multi-channel pipettor for dispensing buffers and removing supernatant.
 - Use 10 µl of 20X TiO₂ Magnetic Beads per sample. This volume may be adjusted; however, for best results use a ratio of 1 µl of stock beads to 10 µg of peptide mixture.
 - To minimize bead loss, leave up to 10 µl of liquid in the wells when removing buffers; however, for the last wash step, completely remove the Washing Buffer from the bead before adding Elution Buffer.
1. (Optional) Before use, rinse all plastic wear (such as the 96-well plates) three times with acetonitrile to minimize contamination from polymers leaching from the plastics.
 2. Multiply the total number of samples plus one by 10 µl to determine the total volume of magnetic beads required. Place the determined volume of 20X TiO₂ Magnetic Beads into a 50 ml polypropylene conical tube.
Note: To ensure homogeneity, mix the vial of 20X TiO₂ Magnetic Beads thoroughly before use by repeated inversion, gentle vortexing or rotating platform.
 3. Add 190 µl of prepared Binding Buffer for every 10 µl of beads dispensed in Step 2. Screw the cap onto the tube and invert several times to ensure complete suspension of the beads.
 4. Pipette 200 µl of the bead suspension into each well of the provided 96-well PCR plate.
Note: Periodically mix the bead suspension during dispensing to prevent settling.
 5. Place the 96-well plate onto a magnetic plate separator for 1 minute. The beads will collect in the O-ring near the bottom of each well. Use a multi-channel pipettor to remove the supernatant from the center of the wells.
 6. Remove the 96-well plate from the magnet. Add 200 µl per well of prepared Binding Buffer to the beads. Do not pipette the beads up and down. Add Binding Buffer quickly to suspend the beads.
 7. Repeat Steps 5 and 6 twice.

D. Manually Perform the Phosphopeptide Enrichment

Note: For best results, desalt the sample peptide mixture on an SPE column or a 96-well device before processing with the TiO₂ Magnetic Beads. See the Additional Information Section for protocol. Samples eluted from SPE plates or columns in 80% acetonitrile/0.1% formic acid may be directly applied to beads after adding formic acid to a final concentration of 2%.

Note: If sample peptide mixture is not eluted from an SPE device, ensure that the peptide mixture is in a final solution of 80% acetonitrile/2% formic acid before applying to the TiO₂ Magnetic Beads.

1. Remove the 96-well plate from the magnet. Add 100 µl of sample peptide mixture per well to the beads and pipette up and down to mix.
2. Place the plate on a magnetic plate separator for 1 minute. Use a multi-channel pipettor to remove the supernatant from the center of the wells.
3. Remove the plate from the magnet and add 200 µl of Binding Buffer (per well) quickly to resuspend the beads. Place the plate on a magnetic plate separator for 1 minute and remove the supernatant. Repeat this step three times.
4. Remove the 96-well plate from the magnet and add 200 µl of Washing Buffer (per well) quickly to resuspend the beads.
5. Place the plate on a magnetic plate separator for 1 minute and remove the supernatant. Completely remove the Washing Buffer before proceeding to the elution step.
6. Remove the plate from the magnet and add 30 µl of Elution Buffer (per well) and pipette up and down a few times to ensure complete coating of the beads with buffer. Incubate at room temperature for 10 minutes.
7. Place the plate on a magnetic plate separator for 1 minute and remove the supernatant containing eluted phosphopeptides.

Note: Avoid aspirating the beads with the eluates. Beads may interfere with mass spectrometry analysis.

8. Transfer samples to the unused 96-well PCR plate provided and dry in a vacuum concentrator. For LC-MS analysis using reverse-phase chromatography (e.g., C18), suspend sample in 5% acetonitrile/1% formic acid in water. Alternatively, for MALDI analysis suspend in a matrix of α -cyano-4-hydroxycinnamic acid dissolved in 3% formic

acid/50% acetonitrile (prepare on the same day of use). Empirically determine the concentration of MALDI matrix to use. The optimal concentration depends on MS instrument used and final concentration of peptides in sample.

Note: Covering the matrix after spotting with 1 μ l of 25 mM ammonium citrate may enhance detection of phosphopeptides in MALDI-MS analysis.

Procedure for Automated Enrichment of Phosphopeptides on the KingFisher PCR Plate

A. Additional Materials Required (see the Related Products Section for ordering information)

- KingFisher Flex or KingFisher 96 Instrument with PCR Magnet Head
- KingFisher Flex 96 Tip Comb for PCR Head Magnets
- Thermo Scientific Thermo-Fast 96 Robotic PCR Plate, 0.2 ml well volume
- Sample peptide mixture
- 50 ml polypropylene conical centrifuge tubes
- 15 ml polypropylene conical centrifuge tubes
- Acetonitrile
- Formic Acid
- Vacuum concentrator with a 96-well plate rotor

B. Prepare the KingFisher Instrument

Note: The following protocols are designed for general use with the KingFisher Flex or KingFisher 96 Instruments. If desired, the protocols can be modified as needed using the BindIt™ Software provided with the instrument.

1. Download the “Phosphopeptide Enrichment PCR Plate” protocol from the web site (www.thermo.com/kingfisher) into the BindIt Software on an external computer.
2. Transfer the protocol to the KingFisher Instrument from an external computer. See BindIt Software User Manual for detailed instructions on importing protocols.

C. Prepare the Binding and Washing Buffers

Note: Prepare buffers just before each experiment.

- | | |
|----------------|---|
| Binding Buffer | Multiply the number of samples by 510 μ l to determine the total buffer volume required. Add 5% for excess. Combine equal volumes of acetonitrile and Binding Buffer in a polypropylene conical tube (15 ml or 50 ml) and mix to obtain a homogeneous solution. Close tube tightly to prevent acetonitrile evaporation. |
| Washing Buffer | Multiply the number of samples by 130 μ l to determine the total buffer volume required. Add 5% for excess. Combine equal volumes of acetonitrile and Washing Buffer in a 15 ml polypropylene conical tube and mix to obtain a homogeneous solution. Close tube tightly to prevent acetonitrile evaporation. |

D. Prepare the TiO₂ Magnetic Beads and Plate Set-up

Note: The following protocol is for processing less than 1 mg of peptide mixture. The protocol uses 10 μ l of 20X TiO₂ Magnetic Beads per sample. This volume can be adjusted; however, for best results, use a ratio of 1 μ l of stock beads to 10 μ g of peptide mixture.

1. (Optional) Rinse all plastic wear (such as the 96-well plates) three times with acetonitrile to minimize contamination from leached polymers.
2. Multiply the total number of samples plus one by 10 μ l to determine the total volume of magnetic beads required. Place calculated volume of 20X TiO₂ Magnetic Beads into a 15 ml polypropylene conical tube.

Note: To ensure homogeneity, mix the vial of 20X TiO₂ Magnetic Beads thoroughly before use by repeated inversion, gentle vortexing or rotating platform.

3. Add 120 μ l of prepared Binding Buffer for each 10 μ l of beads dispensed in Step 2. Screw the cap onto the tube and invert several times to ensure complete suspension of the beads.
4. Set up the plates according to Table 1, which will subsequently be placed into a KingFisher Instrument.

Table 1. Pipetting instructions for the Phosphopeptide Enrichment PCR Plate Protocol using the Thermo-Fast 96 Robotic PCR Plates.

<u>Plate #</u>	<u>Plate Name</u>	<u>Content</u>	<u>Volume (μl)/well</u>
1	Tip Plate	KingFisher Flex 96 Tip Comb for PCR Magnet Head	-
2	Beads	TiO ₂ beads in Binding Buffer	130
3	Bead Wash	Binding Buffer	130
4	Sample	Sample peptide mixture in 80% acetonitrile/2% formic acid	100
5	Sample Wash	Binding Buffer	130
6	Sample Wash	Binding Buffer	130
7	Sample Wash	Washing Buffer	130
8	Elution	Elution Buffer	30

E. Perform the Phosphopeptide Enrichment PCR Plate Protocol

1. Select the protocol using the arrow keys in the instrument keypad and press Start. See KingFisher User Manual for detailed information.
2. Slide open the door of the protective cover on the instrument. Load plates 1-8 into the instrument according to the protocol request and confirm each action by pressing *Start*. Place each plate in the same orientation.
3. Press *Start* to initiate the protocol.
4. After the samples are processed, remove the plates as instructed by the display on the instrument. Press *Start* after removing each plate.
5. Press *Stop* after all plates are removed.
6. Transfer samples to a new PCR plate and lyophilize to dryness using a vacuum concentrator.
7. For LC-MS/MS analysis, rehydrate each sample in 5% acetonitrile/1% formic acid in water and inject each sample directly from the 96-well plate. Alternatively, for MALDI analysis resuspend samples in α -cyano-4-hydroxycinnamic acid MALDI matrix solution and spot onto a MALDI chip.

Procedure for Automated Enrichment of Phosphopeptides using Deep-well Plates

A. Additional Materials Required (see the Related Products Section for ordering information)

- KingFisher Flex or KingFisher 96 Instrument with 96 Deep Well Head
- Microtiter Deep Well 96 Plate, V-bottom, polypropylene
- KingFisher Flex 96 tip comb for Deep Well magnets
- Sample peptide mixture
- 50 ml polypropylene conical centrifuge tubes
- 15 ml polypropylene conical centrifuge tubes
- Acetonitrile
- Formic acid
- Vacuum concentrator with a 96-well plate rotor

B. Prepare the King Fisher Instrument

Note: The following protocol is for general use with the KingFisher Flex or KingFisher 96 Instrument. Modify the protocol as needed using the BindIt Software provided with the instrument.

1. Download the “Phosphopeptide Enrichment Deep Well” protocol from the web site (www.thermo.com/kingfisher) into the BindIt Software on an external computer.
2. Transfer the protocol to the KingFisher Instrument from the external computer. See BindIt Software User Manual for detailed instructions on importing protocols.

C. Prepare the Binding and Washing Buffers

Note: Prepare buffers just before each experiment.

- Binding Buffer** Multiply the number of samples by 3.9 ml to determine the total buffer volume required. Add 5% for excess. Combine equal volumes of acetonitrile and Binding Buffer in a 50 ml polypropylene conical tube and mix to obtain a homogeneous solution. Close the tube tightly to prevent acetonitrile evaporation.
- Washing Buffer** Multiply the number of samples by 1 ml to determine the total buffer volume required. Add 5% for excess. Using this number, combine equal volumes of acetonitrile and Washing Buffer in a 15 ml polypropylene conical tube and mix to obtain a homogeneous solution. Close the tube tightly to prevent acetonitrile evaporation.

D. Prepare the TiO₂ Magnetic Beads

Note: The following protocol is recommended when processing a minimum of 1 mg of peptide mixture. The protocol uses 100 µl of 20X TiO₂ Magnetic Beads per sample. This volume can be adjusted; however, a ratio of 1 µl of stock beads to 10 µg of peptide mixture is recommended.

1. (Optional) Rinse all plastic wear to be used (such as the 96-well plates) three times with acetonitrile to minimize contamination from polymers leaching from the plastics.
2. Determine the total number of samples to be processed. Multiply the total number of samples plus 0.5 by 100 µl to calculate the total volume of magnetic beads required. Place the calculated volume of 20X TiO₂ Magnetic Beads into a 15 ml polypropylene conical tube.

Note: To ensure homogeneity, mix the vial of 20X TiO₂ Magnetic Beads thoroughly before use by repeated inversion, gentle vortexing or rotating platform.

3. Add 900 µl of prepared Binding Buffer per sample (+ 0.5) to the tube. Screw the cap onto the tube and invert several times to ensure complete suspension of the beads.
4. Set up the plates according to Table 2 which will subsequently be placed into the KingFisher Instrument.

Table 2. Pipetting instructions for the Phosphopeptide Enrichment using the Microtiter Deep Well 96 Plates.

<u>Plate #</u>	<u>Plate Name</u>	<u>Content</u>	<u>Volume (µl)/well</u>
1	Tip Plate	KingFisher Flex 96 Tip Comb for Deep Well Magnets	-
2	Beads	TiO ₂ beads in Binding Buffer	1,000
3	Bead Wash	Binding Buffer	1,000
4	Sample	Sample peptide mixture in 80% acetonitrile/2% formic acid	1,000
5	Sample Wash	Binding Buffer	1000
6	Sample Wash	Binding Buffer	1,000
7	Sample Wash	Washing Buffer	1,000
8	Elution	Elution Buffer	150

E. Perform the Phosphopeptide Enrichment Deep-well Plate Protocol

1. Select the protocol using the arrow keys in the instrument keypad and press *Start*. See the KingFisher User Manual for detailed information.
2. Slide open the door of the instrument's protective cover. Load plates 1 through 8 into the instrument according to the protocol request, placing each plate in the same orientation. Confirm each action by pressing *Start*.
3. Press *Start* to initiate the protocol.
4. After the samples are processed, remove the plates as instructed by the display on the instrument. Press *Start* after removing each plate.
5. Press *Stop* after all plates are removed.
6. Transfer samples to a new PCR plate and lyophilize to dryness using a vacuum concentrator.
7. For LC-MS/MS analysis, rehydrate each sample in 5% acetonitrile/1% formic acid in water and inject each sample directly from the 96-well plate. Alternatively, for MALDI analysis resuspend samples in α -cyano-4-hydroxycinnamic acid MALDI matrix solution and spot onto a MALDI chip.

Troubleshooting

Problem	Possible Cause	Solution
Lack of phosphopeptides identified	Dephosphorylation occurred during isolation and processing	Include phosphatase inhibitors during sample preparation
	Poor cell health or improper stimulation to induce phosphorylation events	Check cell health and growth and confirm stimulation by Western blot
	Low amount of phosphorylation events in the sample	Increase amount of starting material being processed
	Interference from inorganic phosphates or highly buffered starting material prevented binding buffer from adjusting sample to the required conditions	Use phosphate-free buffers, dilute sample with binding buffer or change sample preparation method
	Improper MS instrument setting	Use control phosphopeptides (e.g., digested α -casein) to determine optimal instrument settings
	Neutral loss of phosphate groups causing poor MS/MS phosphopeptide sequencing	Perform neutral loss scan and MS ³ experiments or, on ETD-equipped instruments, use ETD fragmentation to preserve phosphorylation events
	Sample digestion incomplete	Monitor sample digestion by SDS-PAGE
Identification of a large numbers of non-phosphorylated peptides	Sample contains highly acidic peptides	Methylate sample peptide mixture before processing (see Additional Information Section)
	Insufficient washing	Perform an additional wash
	Highly buffered starting material prevented the binding buffer from adjusting sample to the required conditions	Dilute sample with binding buffer or change sample preparation method

Additional Information

- Visit www.thermo.com/pierce for additional information including the following:
 - Tech Tip #43: Protein Stability and Storage
- Visit www.thermo.com/kingfisher for information on KingFisher Products.
- In the U.S.A., purchase KingFisher Supplies from VWR. Contact your local Thermo Fisher Scientific office to purchase KingFisher Supplies outside the U.S.A.

A. Procedure for Methylation of Carboxyl Groups

Note: The presence of significant amounts of highly acidic peptides may hinder phosphopeptide enrichment and detection because of carboxyl groups interacting with the TiO₂ surface. To minimize this interaction, methylate the carboxyl groups before phosphopeptide enrichment. Methylation is not recommended for all samples as nonspecific binding is typically low and methylation significantly increases the difficulty of data analysis.

1. Lyophilize a peptide sample and dissolve it in 1 ml of 3 N methanolic HCl. Incubate the reaction for 60 minutes at room temperature.

Note: Use prepared 3 N methanolic HCl that is provided in glass ampules (e.g., Sigma Product No. 32355).

2. Lyophilize the sample to remove the solvent.
3. Dissolve the peptides in up to 1 ml of 80% acetonitrile/0.1% formic acid and proceed to enrichment of phosphopeptides using the Pierce Magnetic TiO₂ Phosphopeptide Enrichment Kit.

B. Sample Concentration and Desalting Using Solid Phase Extraction Cartridges or 96-Well Plates

Note: Sample concentration and desalting is recommended before enrichment with the Pierce Magnetic TiO₂ Phosphopeptide Enrichment Kit. To process up to 2 mg of peptides, use the Thermo Scientific HyperSep SPE Columns or Thermo Scientific Hypersil C18 96-well Plates (see the Related Products Section for ordering information).

1. Rinse each SPE cartridge or well with one full volume of acetonitrile.
2. Rinse each SPE cartridge or well with one full volume of 0.25% TFA in water. Repeat this step twice.
3. Load peptide digest sample onto SPE cartridge or wells at a flow rate of 1 ml per minute.
4. Rinse each SPE cartridge or well with one full volume of 0.25% TFA in water. Repeat this step four times
5. Elute the desalted peptide samples in 80% acetonitrile/0.1% formic acid.
6. Add formic acid to eluted peptide sample to a final concentration of 2%. Add samples directly to TiO₂ beads.

Related Products

Products available from www.thermo.com/pierce

28905	Formic Acid, 10 × 1 ml ampules
21358	MagnaBind™ Magnet for 96-Well Separator
21357	MagnaBind Magnet for one 1.5 ml Microcentrifuge Tube
21359	MagnaBind Magnet for 6 Microcentrifuge Tubes
88800, 88801	Pierce Protein A Magnetic Beads
88806, 88807	Pierce Protein G Magnetic Beads
88821, 88822	Pierce Glutathione Magnetic Beads

Products available from www.thermo.com

SC250EXP-115	Express SpeedVac Concentrator SC250
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Products available from www.thermo.com/abgene

AB1300	Thermo-Fast 96 Robotic PCR Plate, 0.2 ml well volume
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Products available from www.fishersci.com

14-432-22	50 ml Polypropylene Conical Centrifuge Tubes
14-959-49B	15 ml Polypropylene Conical Centrifuge Tubes
A955-1	Acetonitrile, 1 L
60108-304	HyperSep SPE Columns
60300-428	Hypersil C18 96-well Plates

Products available from www.vwr.com

5400630	KingFisher Flex with 96 Deep Well Head Instrument
97002514	KingFisher Flex 96 Tip Comb for PCR Head Magnets
95040450	Microtiter Deep Well 96 Plate, V-bottom, polypropylene
97002534	KingFisher Flex 96 Tip Comb for Deep Well Magnets

Reference

1. Hubbard, M.J. and Cohen, P. (1993). On target with a new mechanism for the regulation of protein phosphorylation. *Trends Biochem.* **18**:172.

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Current versions of product instructions are available at www.thermo.com/pierce. For a faxed copy, call 800-874-3723 or contact your local distributor.

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