INSTRUCTIONS

Pierce® Phosphoprotein Enrichment Kit

90003 2021.1

Number	Description
90003	**Pierce Phosphoprotein Enrichment Kit**
	Kit Contents:
	Phosphoprotein Enrichment Column, 10 each, 1mL resin bed
	Lysis/Binding/Wash Buffer, 325mL
	Elution Buffer, 60mL
	CHAPS, 1g
	White Column Caps, 10 each
	Pierce Concentrator, 7mL/9K, 10 devices
	Storage: Upon receipt store at 4°C. Product shipped at ambient temperature.

Introduction

The Thermo Scientific Pierce Phosphoprotein Enrichment Kit enables efficient enrichment of phosphorylated proteins derived from mammalian cells in culture and tissue. The column contains a proprietary metal/buffer composition that produces superior yields with negligible nonspecific binding. For convenience the Pierce Phosphoprotein Enrichment Kit incorporates a spin format that produces enriched phosphoproteins within 2 hours. Once enriched, the phosphoproteins can be used for downstream proteomic analyses by Western blotting, mass spectrometry, 2D-PAGE and protein arrays.

Important Product Information

- Adding CHAPS (0.25%) to the Lysis/Binding/Wash Buffer is required and helps maintain protein solubility. However, adding CHAPS to the Elution Buffer is optional and NOT recommended if performing mass spectrometry downstream.
- Typical yields are 10-25% of the total cellular protein loaded onto the column. Factors that influence yield include cell type and cell treatment (pharmacological agents, growth factors, etc.).
- Specific binding of phosphoproteins is dependent upon protein isoelectric point (pI) and buffer pH. If required, adjust the pH of the Lysis/Binding/Wash Buffer or Elution Buffer to enrich for different populations of phosphoproteins.
- The Elution Buffer consists of 75mM sodium phosphate, 500mM sodium chloride; pH 7.5. Therefore, samples must be desalted before performing 2D gel analysis.
- The Lysis/Binding/Wash and Elution Buffers contain 0.01% sodium azide as an antimicrobial.
- Tissues must be homogenized in appropriate buffer, centrifuged and diluted 1:1 in Lysis/Binding/Wash Buffer before loading the sample onto a Phosphoprotein Enrichment Column in Section B.

Additional Materials

- EDTA-free protease inhibitors such as Thermo Scientific Halt Protease Inhibitor Single-Use Cocktail EDTA-Free (Product No. 78425)
- Phosphatase inhibitors such as Halt™ Phosphatase Inhibitor Cocktail (100X), (Product No. 78420)
- Phosphate-free buffer such as 50mM HEPES, pH 7.0
- 50mL conical tubes

- Centrifuge with swinging-bucket or fixed-angle rotor for 15mL and 50mL conical tubes, rated for ≥ 2000 × *g*
- Thermo Scientific T-PER Tissue Protein Extraction Reagent (Product No. 78510) (For tissue lysis protocol)
- Optional: DNase I (5000-10,000 units/mL) (Product No. 89835)
- Optional: PMSF (Product No. 36978)

Material Preparation

10% CHAPS Solution	Add 10mL of ultrapure water to the bottle containing 1g of CHAPS. Mix by inversion and vortex. Store solution at 4°C.
Lysis/Binding/Wash Buffer with CHAPS (0.25%)	Prepare enough Lysis/Binding/Wash Buffer with a final concentration of 0.25% CHAPS for each step, typically 24mL for a 2mg load.
Elution Buffer with CHAPS (0.25%)	If performing downstream mass spectrometry applications DO NOT add CHAPS to the Elution Buffer. Otherwise, to maintain protein solubility, prepare 5mL of Elution Buffer by adding 125µL of 10% CHAPS for each column being used.

Procedure for Enrichment of Phosphoproteins

Note: For cell lysis, use Section A and continue to Section C. For tissue lysis, proceed to Section B and continue with Section C, protocol step 2.

A. Cell Lysis

1. Wash cells 2X with a non-phosphate-based buffer (e.g., 50mM HEPES, pH 7.0). Remove HEPES wash buffer.
2. Lyse cells by adding 1mL (15cm plate) of Lysis/Binding/Wash Buffer with CHAPS, 1X Halt Protease Inhibitor EDTA-free and 1X Halt Phosphatase Inhibitor Cocktail to each cell culture plate.
3. Scrape cells into appropriate-sized collection tube and place cells on ice for 45 minutes, vortexing periodically.
4. Centrifuge lysed cells at 10,000 × *g* for 20 minutes at 4°C to pellet cellular debris.
5. Collect supernatant and proceed to the next section, or freeze sample at -80°C for later use.

B. Tissue Lysis

1. Wash tissue two times with a sufficient volume of non-phosphate-based buffer (e.g., 50mM HEPES, pH 7.0) to completely cover the tissue. Remove wash buffer.
2. Weigh out tissue; for every 1gm of tissue, add 20mL of T-PER® Reagent or 10-20mL of RIPA Buffer containing 1X Halt Protease Inhibitor EDTA-free and 1X Halt Phosphatase Inhibitor Cocktail.
3. Using a polytron or tissue homogenizer, process tissue until homogeneous throughout.
4. Centrifuge the sample at 10,000 × *g* for 5 minutes to pellet tissue debris.
5. Collect supernatant and proceed to the next step, or freeze sample at -80°C for later use.
6. Prior to Phosphoprotein Enrichment, determine the protein concentration of the supernatant by performing a protein assay.
7. Adjust the concentration of sample to 0.5mg/mL with Lysis/Binding/Wash Buffer. Up to 4mg of total protein may be applied to each column. Add a volume of Lysis/Binding/Wash Buffer that is at least equal to the volume of the sample; larger volumes can also be added if greater dilution is required.

C. Phosphoprotein Enrichment

1. Determine the protein concentration of the supernatant by performing a protein assay. Adjust the concentration to 0.5mg/mL with Lysis/Binding/Wash Buffer. Up to 4mg of total protein may be applied to each column.
2. Invert column to suspend the resin slurry.
3. Snap off bottom of column and loosen cap. Place column into a 50 ml conical tube.

4. To remove the storage solution, centrifuge column at 1000 × *g* for 1 minute at 4°C and discard the flow-through.
5. Equilibrate resin by adding 5mL of Lysis/Binding/Wash Buffer with CHAPS. Place column into a 50mL conical tube, centrifuge at 1000 × *g* for 1 minute at 4°C and discard the flow-through.
6. Plug bottom of column and add diluted lysate. Screw cap on top of column and invert several times to mix. Place on a platform rocker for 30 minutes at 4°C.

   Note: The presence of protease and phosphatase inhibitors might make the resin slightly yellow; however, this color change has not adversely affected results.
7. Remove bottom plug and place column in a 50mL conical tube. Centrifuge column at 1000 × *g* for 1 minute at 4°C. If desired, save the flow-through for downstream analysis.
8. Wash resin by adding 5mL Lysis/Binding/Wash Buffer with CHAPS. Place column into a 50mL conical tube and centrifuge at 1000 × *g* for 1 minute at 4°C. If desired, save the wash fraction for downstream analysis.
9. Repeat Step 7 two times for a total of three washes.
10. Plug bottom of column and transfer to a new 50mL conical tube. Add 1mL of Elution Buffer (with optional CHAPS) and incubate at room temperature with occasional agitation for 3 minutes.
11. Remove plug and centrifuge at 1000 × *g* for 1 minute at 4°C.
12. Repeat elution steps four times for a total of five elution fractions (5mL total). Pool the fractions and proceed to the next section or freeze sample at -80°C for later use.

D. Phosphoprotein Concentration

Note: The Pierce Concentrators can be used effectively at a relative centrifugal force of 2000-9000 × *g*; the recommended speeds are 2500-4500 × *g* for swinging-bucket rotors and 5000-7000 × *g* for fixed-angle rotors. Set centrifuge to the appropriate speed at 4°C.

1. Place sample into upper sample chamber of concentrator. Maximum sample volume is 7mL using a swinging bucket rotor and approximately 4.5-5.0mL using a fixed-angle rotor. The pooled elution fractions will be approximately 5mL.
2. To prevent over-concentrating, control the dead-stop volume by removing cap and upper chamber and placing 2.5mL of ultrapure water in the lower chamber. Replace the upper chamber and add the eluate (from Step B.11).
3. Cap and place concentrator assembly into rotor with proper counterbalance.
4. Centrifuge for approximately 30 minutes or until the sample is 150-200µL. Remove concentrated protein promptly.
5. Use a 200µL or similar pipette tip to gently aspirate concentrated sample from upper chamber.

Troubleshooting

Problem	Possible Cause	Solution
Low yield	Cell harvest conditions were not optimal	Before harvest, wash cells 2X with a non-phosphate-based buffer such as 50mM HEPES, pH 7.0
	Column was not equilibrated with Binding Buffer	Equilibrate column with 5mL (5X bed volume) of Lysis/Binding/Wash Buffer with CHAPS
	Sample was not equilibrated in Binding Buffer	Dilute the sample to 0.25-0.5mg/mL total protein in Lysis/Binding/Wash Buffer with CHAPS
	Sample was too concentrated	Use a sample total protein concentration from 0.25 to 0.5mg/mL
	Sample was too viscous	Add DNAse to the Lysis/Binding/Wash Buffer
	Insufficient sample/resin mixing	Ensure that sample mixes well at 4°C on a rocking platform for 30 minutes
	Chelating/reducing agent was present in sample	Buffer exchange the sample to eliminate chelating agents such as EDTA, or reducing agents such as β-mercaptoethanol or DTT before column chromatography

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Nonspecific protein contamination	Column was overloaded	For best results, use ≤ 4mg of total cellular protein at ≤ 0.5mg/mL
	Inadequate washing	Make sure to wash the column 3X for a total of 15mL using the Lysis/Binding/Wash Buffer with CHAPS
2D smearing	Salt was present	Perform a buffer exchange before 2-D analysis

Related Thermo Scientific Products

89884 **Pierce Concentrator, 7mL/9K**, 25 devices

89885 **Pierce Concentrator, 20mL/9K**, 25 devices

78425 **Halt Protease Inhibitor Single-Use Cocktail EDTA-Free**, 100 µl × 24 microtubes

78420 **Halt Phosphatase Inhibitor Cocktail (100X)**, 1mL

23236 **Pierce Coomassie Plus (Bradford) Assay Kit**

23227 **Pierce BCA Protein Assay Kit**

23208 **Pre-Diluted Protein Assay Standards: Bovine Serum Albumin (BSA) Set**

34080 **SuperSignal® West Pico Chemiluminescent Substrate**

46428 **Restore™ PLUS Western Blot Stripping Buffer**, 30mL

34090 **CL-XPosure™ Film,** 5 × 7in (13 × 18cm), 100/pkg

89983 **Pierce SILAC Protein Quantitation Kit – DMEM**

89982 **Pierce SILAC Protein Quantitation Kit – RPMI 1640**

89893 **Zeba™ Spin Desalting Columns, 10mL,** 5 columns

78510 **T-PER Tissue Protein Extraction Reagent,** 500mL

89900 **RIPA Buffer,** 100mL

89901 **RIPA Buffer,** 250mL

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