Pro-Q® Diamond Phosphoprotein Enrichment Kit (P33358)

Quick Facts

Storage upon receipt:

- Store Phosphoprotein Enrichment Module (P33361) at 2–6°C; DO NOT FREEZE
- Store protease inhibitor at ≤–20°C
- Store endonuclease at –20°C; DO NOT STORE at –70°C

Introduction

The Pro-Q® Diamond Phosphoprotein Enrichment Kit enables efficient, nonradioactive isolation of phosphoproteins from complex cellular extracts. The kit provides resin, reagents, and columns designed to isolate phosphoproteins from 0.5–1.0 mg of total cellular protein per column. The column bed volume can be easily scaled up or down depending on the amount of available starting material. The phosphoprotein-binding properties of the resin allow efficient capture of both native and denatured proteins. Therefore, cell or tissue samples can be denatured in lysis buffers and stored in the freezer prior to the phosphoprotein enrichment procedure. Protocols for both undenatured and denatured lysates are provided. The procedures can be completed in approximately 3 hours.

Materials

Kit Contents

Phosphoprotein Enrichment Module (P33361)

- **Resin**, 6 mL (12 mL of 50% v/v slurry)
- **Columns**, 2 mL disposable, 10
- **Lysis buffer**, 20 mL
- **Wash buffer**, 250 mL
- **Elution buffer**, 30 mL
- **Vivaspin® filtration concentrators**, with 10 kDa cutoff polyethersulfone membrane, 10

Protease Inhibitor and Endonuclease Module (P33360)

- **Protease inhibitor**, 200 µL
- **Endonuclease**, 100 µL of 25 U/µL

Storage and Handling

Immediately upon receipt, store the Protease Inhibitor and Endonuclease Module (P33360) at ≤–20°C and the Phosphoprotein Enrichment Module (P33361) at 2–6°C. Do not store the endonuclease at –70°C. Do not freeze the Phosphoprotein Enrichment Module. The disposable columns and Vivaspin® filtration concentrators may also be stored at room temperature. After thawing the protease inhibitor for the first time, dispense it into 20–25 µL aliquots and store them at ≤–20°C to minimize multiple freeze/thaw cycles.

Materials Not Provided

- Isopropanol or ethanol
- 25 mM Tris, pH 7.5, 0.25% CHAPS
- 50 mM Tris, pH 8.0, 2% SDS, 10 mM EDTA, 5 mM TCEP (optional, for step 4.4)
- Methanol
- Chloroform
- EZQ® Protein Quantitation Kit (R33200) or other protein quantitation method
- Deionized water

Procedure

This procedure describes loading 0.5–1 mg of nondenatured or denatured cell lysate per 500 µL of packed resin. Under the conditions described, very little phosphoprotein will be present in the flow-through (Figure 1). The yield of phosphoprotein in the eluate is typically about 10% of the amount of total protein loaded. Larger amounts of protein can be loaded to increase the absolute amount of phosphoprotein recovered in the eluate; however, phosphoprotein will be present in the flow-through as column capacity is exceeded, and the percentage of phosphoprotein recovered will be reduced.

Prepare Column

1.1. Snap off the end tab of a disposable column. Add 200 µL of isopropanol or ethanol to wet the column filter.

1.2. Gently swirl the resin until it is uniformly suspended. Cut 3 mm from the end of a 1 mL disposable pipet tip to create a wider bore that will allow accurate dispensing of the resin. Pipet 1 mL of the well-suspended slurry into the column, and allow the liquid to flow to waste.

1.3. Add 1 mL of deionized water to the column two times, and discard the flow-through. Cap the bottom and top of the column and set aside while the lysate is prepared.

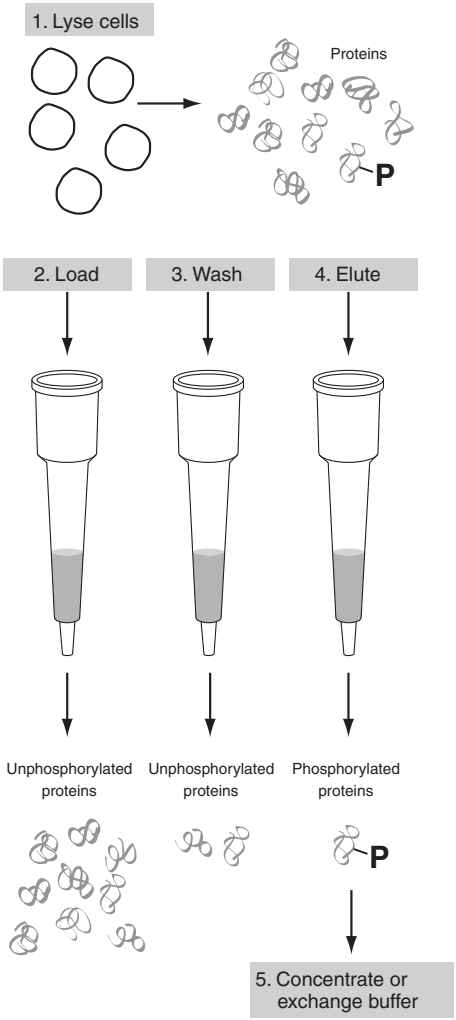

Figure 1. Pro-Q® Diamond Phosphoprotein Enrichment Kit procedure. A clarified cell lysate is loaded onto a column containing a phosphoprotein-binding resin. Unphosphorylated proteins flow through the column and are washed out. Phosphorylated proteins are subsequently eluted.

Prepare Lysate

Nondenatured lysate:

2.1 Using a phosphate-free buffer such as HEPES, prepare a cell pellet that would yield approximately 0.5–1 mg of total protein. In our experience, 2×10^7 Jurkat cells yield about 1 mg of solubilized protein.

2.2 Thaw the endonuclease and protease inhibitor. Add 500 µL of lysis buffer, 10 µL of endonuclease, and 5 µL of protease inhibitor to the cell pellet. Pipet up and down to disperse the pellet, then incubate on ice for 30 minutes, vortexing for 1 minute every 5–10 minutes during the incubation period. If undigested DNA (indicated by the presence of a gelatinous mass) persists after the first 15 minutes of incubation, it may be removed by addition of more endonuclease or by sonication with a probe sonicator.

2.3 After the 30 minute incubation, centrifuge the cell lysate at 10,000 × g and 4°C for 20 minutes. Transfer the supernatant to a clean tube, and determine the protein concentration directly using the EZQ® Protein Quantitation Kit or another method. Ideally, the concentration should be between 1 and 2 mg/mL.

2.4 Save 50 µL of the clarified lysate for later SDS-PAGE analysis. Precipitate it as described in step 6.2, using one third of the volumes of methanol, chloroform, and water specified, to preserve the scale.

2.5 Dilute the remaining 450 µL of clarified lysate to 5 mL with wash buffer. The final concentration should be 0.1–0.2 mg/mL.

Denatured lysate:

3.1 Follow steps 2.1 through 2.3 of the lysis procedure for nondenatured lysate.

3.2 Precipite the clarified lysate as described in the section *Precipitate Sample* below, starting at step 6.2. Store the precipitated pellet at ≤–20°C until ready to perform the phosphopeptide enrichment.

3.3 Solubilize the precipitated cell protein pellet in urea-based 2-D electrophoresis sample buffer at a concentration of 1–2 mg/mL. Avoid buffers with phosphate, SDS, or other anionic detergents. The pellet may require vigorous vortexing or sonication to maximize solubilization of the precipitated protein.

3.4. After solubilization, centrifuge the cell lysate at 10,000 × g for 5 minutes and recover the supernatant. Save 50 µL of the clarified lysate for later SDS-PAGE analysis, and precipitate it as described in section 6.2, using one third of the volumes of methanol, chloroform, and water specified, to preserve the scale. If the saved sample is to be used for 2-D separation, precipitation is unnecessary since it is already in 2-D electrophoresis sample buffer from step 3.3.

3.5 Dilute the remaining lysate to a final protein concentration of 0.1–0.2 mg/mL with wash buffer.

Run Sample through Column

4.1 Remove the top and bottom caps from a previously prepared column. Equilibrate the column with 2 × 1 mL of wash buffer.

4.2 Apply the diluted lysate to the column, 1 mL at a time. If desired, save 1 mL of the flow-through for analysis of the unphosphorylated proteins. Concentrate and precipitate the flow-through as described below for the eluate.

4.3 Wash the column with 3 × 1 mL of wash buffer.

4.4 Elute the column with 5 × 250 µL of elution buffer, and collect the eluate fractions. The second and third fractions will have the highest phosphoprotein concentration. The fractions may be analyzed separately or pooled prior to concentrating the eluted phosphoproteins.

Note: If denatured protein is compatible with the intended downstream use of the phosphoprotein-enriched sample, elution with 50 mM Tris, pH 8.0, 2% SDS, 10 mM EDTA, 5 mM TCEP will yield higher recovery of phosphoproteins.

Concentrate Sample

5.1 Transfer up to 600 µL of the pooled column eluate to the upper reservoir of a Vivaspin® filtration concentrator. Replace the cap. Insert the concentrator into a centrifuge so that the printed window faces up.

5.2 Centrifuge at no greater than 15,000 × g until the sample volume is reduced to approximately 50 µL (10–15 minutes is usually sufficient).

5.3 Empty the lower filtrate container, and refill the upper reservoir with more of the unconcentrated pooled eluate.

5.4 Concentrate the sample again. Repeat the process until all of the eluate has been concentrated down to approximately 50 µL.

5.5 Empty the filtrate container again. Add 500 µL of 25 mM Tris, pH 7.5, 0.25% CHAPS to the retentate reservoir, and concentrate the sample to a volume of 50–100 µL. Repeat this buffer exchange step twice. The protein concentration of the concentrated eluate typically will be 0.5–1.5 mg/mL. The sample is now ready for procedures requiring nondenatured protein (or denatured protein, if denatured lysate was prepared or if the alternative elution buffer was used in step 4.4). Proceed to the precipitation procedure for preparation for SDS-PAGE or IEF/SDS-PAGE analysis.

Note: At this point, the concentrated sample can be diluted 1:1 with 2X SDS sample buffer and loaded directly onto a gel. However, we recommend precipitating the sample prior to SDS-PAGE or IEF/SDS-PAGE, in order to remove phospholipids and salts that may interfere with phosphoprotein staining or IEF.

Precipitate Sample

6.1 Transfer the concentrated retentate from step 5.5 to a 1.5 mL microcentrifuge tube. To ensure maximum recovery of protein, the retentate reservoir may be rinsed with 50–100 µL of SDS-containing buffer (e.g., SDS-PAGE sample buffer) and the rinse added to the transferred sample. Add enough buffer so that the final volume of the transferred sample is 150–200 µL.

6.2 Precipitate the sample using the methanol-chloroform-water method [1] described below. The volumes given are scaled for a 1.5 mL microcentrifuge tube.

a. Add 600 µL of methanol to the 150–200 µL protein sample in the microcentrifuge tube, and vortex.
b. Add 150 µL of chloroform, and vortex.
c. Add 450 µL of deionized water, and vortex.
d. Centrifuge for 5 minutes at 13,000 × g, then carefully remove and discard as much of the upper aqueous phase as possible while leaving the interface layer containing the protein precipitate intact.
e. Add 450 µL of methanol to the tube, and vortex.
f. Centrifuge for 5 minutes at 13,000 × g to pellet the protein, then remove and discard the supernatant. Leaving the cap open, cover the tube with a lint-free tissue and allow the pellet to air-dry on the bench or in a fume hood, for 1 hour to overnight.
g. Cap the tube and store it in the freezer until use.

Analyze by SDS-PAGE or IEF/SDS-PAGE

7.1 Resolubilize the precipitated sample in SDS-PAGE or IEF/SDS-PAGE sample buffer, aiming for a concentration of approximately 1 mg/mL. The following are some guidelines for achieving a suitable concentration:

a. Lysate: Assuming the clarified lysate contains 1–2 mg/mL protein (see step 2.3), 50 µL of the undiluted lysate can be precipitated directly (see step 2.4) to yield 50–100 µg of protein. Resolubilize the precipitated pellet in 50 µL of desired buffer.
b. Flow-through: Assuming protein is loaded at 0.1–0.2 mg/mL, 1 mL of flow-through can be concentrated and precipitated as described above and resolubilized in 100 µL of desired buffer.
c. Eluate: Assuming 0.5–1 mg of lysate is loaded onto the column and 50–100 µg of phosphoprotein is recovered in the eluate, resolubilize with 50 µL of desired buffer.

7.2 Vortex the samples well to resolubilize the proteins. It may improve resolubilization to let the samples sit 15–30 minutes and then vortex again.

7.3 If the samples are resolubilized in SDS-PAGE buffer, heat them for 10 minutes at 90ºC. Do not heat the samples if they are resolubilized in a urea-based IEF/SDS-PAGE buffer.

7.4 Centrifuge the samples at maximum speed for 5 minutes, and transfer the supernatant to a clean tube.

7.5 Determine the protein concentration of each sample, and load the desired amount on the gel. Suggested loading amounts are 10–20 µg per lane for NuPAGE® 1 mm × 12 well gels and 20–30 µg for NuPAGE® ZOOM® 1 mm IPG gels.

Note: Reagents for SDS-PAGE and IEF/SDS-PAGE sample preparation (detergents, buffer components) do not interfere with the EZQ® Protein Quantitation Kit. The assay's range is 0.02–5 mg/mL, and only 1 µL of sample is required.

7.6 Run the gels according to the desired protocol. Stain with Pro-Q® Diamond phosphoprotein gel stain (P33300) followed by SYPRO® Ruby protein gel stain (S12000) to analyze for phosphoprotein and total protein, respectively (Figure 2).

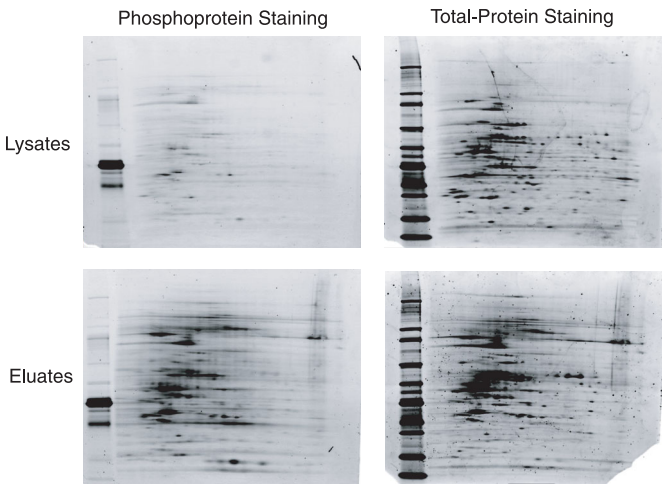

Figure 2. Two-dimensional SDS-PAGE analysis of Jurkat cell lysates before (upper panels) and after (lower panels) phosphoprotein enrichment. Each gel was loaded with 20 µg of protein. The gels were stained with Pro-Q® Diamond phosphoprotein gel stain, then post-stained for total protein with SYPRO® Ruby protein gel stain.

Immunoblotting experiments have shown that the Pro-Q® Diamond Phosphoprotein Enrichment Kit gives better recovery of high molecular weight phosphoproteins than a competitor's kit (Figure 3).

Pro-Q® Diamond phosphoprotein gel stain (P33300) is highly sensitive and selective for phosphoproteins, enabling fast screening without antibodies or radioactivity. PeppermintStick™ phosphoprotein molecular weight standards (P33350), a mixture of phosphorylated and nonphosphorylated proteins, provide accurate size determination as well as a control for detection of phosphorylation. For convenience and added value, the Pro-Q® Diamond Phosphoprotein Enrichment and Detection Kit (P33359) includes the Pro-Q® Diamond Phosphoprotein Enrichment Kit, Pro-Q® Diamond phosphoprotein gel stain, and PeppermintStick™ phosphoprotein molecular weight standards.

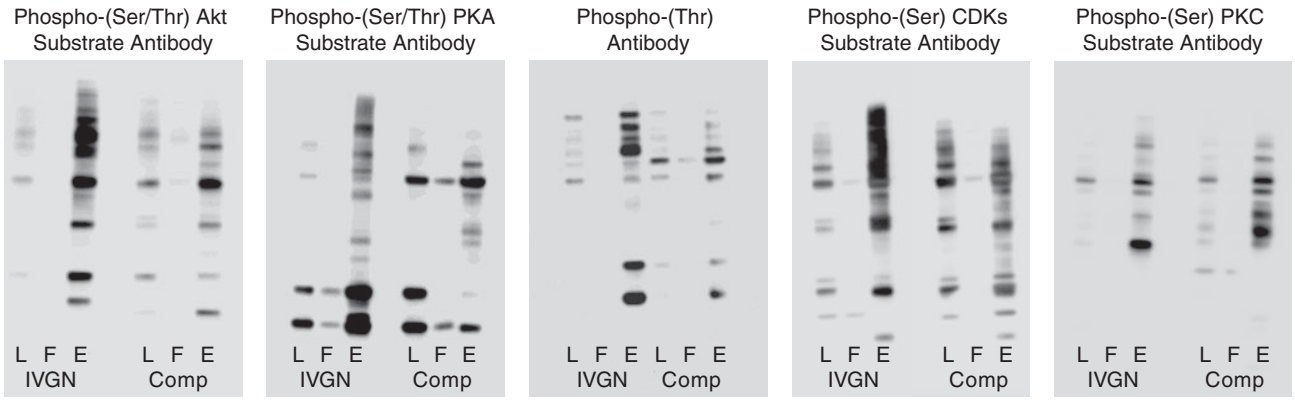

Figure 3. Efficient isolation of high molecular weight phosphoproteins using the Pro-Q® Diamond Phosphoprotein Enrichment Kit. Jurkat cell lysate (L), flow-through (F), and eluate (E) were obtained using the Pro-Q® Diamond Phosphoprotein Enrichment Kit (IVGN) and a competitor's kit (Comp). Protein was precipitated from each fraction and redissolved, and 3 µg of each fraction was separated by SDS-PAGE and immunoblotted. Polyclonal antibodies were obtained from Cell Signaling Technology.

Reference

1. Anal Biochem 138, 141 (1984).

Product List *Current prices may be obtained from our Web site or from our Customer Service Department.*

Cat #	Product Name	Unit Size
P33358	Pro-Q® Diamond Phosphoprotein Enrichment Kit	1 kit
P33359	Pro-Q® Diamond Phosphoprotein Enrichment and Detection Kit	1 kit
P33300	Pro-Q® Diamond phosphoprotein gel stain	1 L
E33201	EZQ® Phosphoprotein Quantitation Kit *2000 assays*	1 kit
R33200	EZQ® Protein Quantitation Kit *2000 assays*	1 kit
P33350	PeppermintStick™ phosphoprotein molecular weight standards *200 gel lanes*	400 µL
P33356	Pro-Q® Diamond Phosphoprotein Blot Stain Kit *20 minigel blots	1 ki
S12000	SYPRO® Ruby protein gel stain	1 L

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