ProteoEnrich™ ATP-Binders™ Kit

About the Kit

ProteoEnrich ATP-Binders Kit	1 kit	71438-3

Description

The ProteoEnrich ATP-Binders Kit is designed for enrichment of protein extracts with ATP-binding proteins (ABPs) such as protein kinases and other nucleotide-binding proteins. The enrichment method is based on the conserved ATP-binding domain of the ABPs. The kit contains a unique polyacrylamide-based resin on which ATP is immobilized by the γ-phosphate through a flexible 13-atom linker (see Figure 1 below). Because ATP immobilization at the γ-position does not interfere with the interactions between the adenine ring or the phosphoryl groups in the α- and β-positions and conserved residues within the ATP-binding pocket, efficient binding of ABPs can occur.

The kit contains a stable, dry polyacrylamide resin, buffers, spin filters, dithiothreitol, activated sodium vanadate, and a protease inhibitor cocktail. Pretreatment of the resin with Resin Conditioning Buffer reduces non-specific binding to the resin and improves the sedimentation properties of the resin. The bind and wash buffers are formulated to further reduce the binding of non-ATP binding proteins by including AMP, ADP, and NADH.

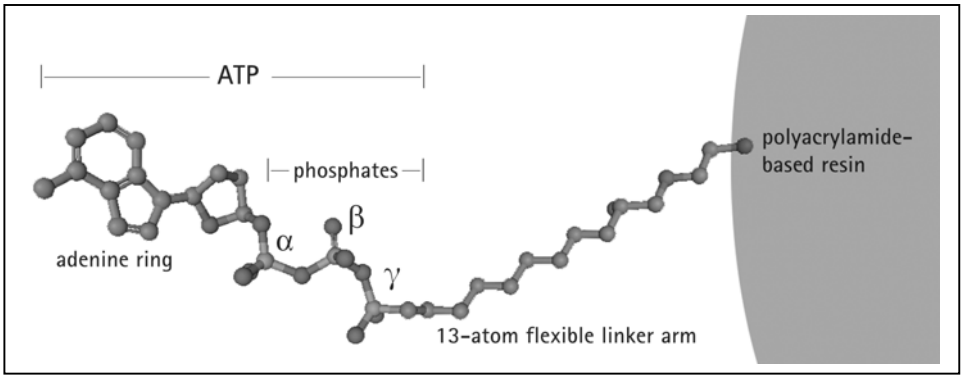

Figure 1. ATP immobilized on polyacrylamide resin

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Table 1 lists the properties of the resin after pretreatment with Resin Conditioning Buffer. The resin exhibits low nonspecific binding and can bind 15–25 µg of ABPs per 100 µl packed resin. It is compatible with a wide range of detergents and suitable for the isolation of cytosolic and solubilized membrane proteins. Typically 3–5 mg of crude cell lysate that has been treated to eliminate endogenous ATP can be loaded onto 100 µl of resin. For more efficient capture of ABPs having relatively weak ATP binding affinities, 1–2 mg of crude extract is recommended. The eluted proteins can be analyzed by electrophoresis or activity assays or used for further purification of individual proteins.

Table 1 Properties of swelled ATP-Binders™ Resin

Particle size	200 µm
Volume (wet resin)	3.5 ml/g (dry weight)
Protein binding	150–250 µg ABPs/ml (packed resin)
Kinase binding	Approximately 250 U*/ml (packed resin)

* Binding capacity determined after resin capture of p60$^{c\text{-}src}$ (Cat. No. PK03). One unit of activity is defined as the amount of enzyme that can transfer one pmol of phosphate per minute from ATP to the tyrosyl residue of Raytide™ Substrate (Cat. No. PK02).

Components

- 100 mg ATP-Binders™ Resin (dry resin, stored under inert gas)
- 1 PBS Tablet
- 2 × 5 ml 100 mM EDTA
- 1.6 ml 1 M DTT
- 2 ml ATP-Binders 6X Resin Conditioning Buffer (25 mM HEPES, 10% Tween®-20, pH 7.2)
- 7 ml ATP-Binders 5X Bind Buffer (300 mM MgCl, 150 mM NaCl, 125 mM HEPES, 0.25% NP-40, pH 7.2)
- 5 ml ATP-Binders Wash Buffer Concentrate (750 mM NaCl, 300 mM $MgCl_2$, 125 mM HEPES, 0.25% NP-40, pH 7.2)
- 1 ml ATP-Binders Elution Buffer Concentrate (125 mM HEPES, 100 mM ATP, 0.25% NP-40, pH 7.2)
- 5 ml ATP-Binders 10X Nucleotide Mix (10X = 10 mM ADP, 10 mM AMP, 10 mM NADH, pH 7.0)
- 1 ml Protease Inhibitor Cocktail Set V, EDTA-Free, Lyophilized
- 2 × 250 µl 100 mM DTT
- 100 µl Activated Sodium Vanadate (200 mM Na_3VO_4, pH 10.0)
- pkg/10 Spin Filter, 2 ml capacity

Storage

Store Spin Filters at room temperature or at 4°C. Store ATP-Binders Resin, ATP-Binders 6X Resin Conditioning Buffer, ATP-Binders 5X Bind Buffer, ATP-Binders Wash Buffer Concentrate, and 100 mM EDTA at 4°C. Store Activated Sodium Vanadate, ATP-Binders 10X Nucleotide Mix, DTT concentrates, ATP-Binders Elution Buffer Concentrate, and Protease Inhibitor Cocktail Set V at –20°C.

ProteoEnrich™ ATP-Binders™ Protocol

The ATP-Binders Resin can be used to capture a representative sample from crude extracts (1). For capture of a representative sample from extracts, do not overload the ATP-Binders Resin. Overloading may result in the displacement of weaker binders by stronger binders and more abundant ATP binding proteins.

The kit does not contain all of the specific substrates or activators that might be necessary for the binding of certain kinases. Appropriate components (i.e., lipids, calcium, manganese) should be added to optimize the binding when required.

Extract preparation

Cell and tissue extracts should be prepared using appropriate buffers that maintain native protein structure and minimize kinase dephosphorylation, to reduce the likelihood of affecting final kinase activity. The ATP-Binders Resin is compatible with the use of detergents and suitable for the isolation of cytosolic and solubilized membrane proteins. For samples that need to be stored longer than 24 hours, we suggest snap freezing extracts in the presence of 10% glycerol, 1 mM DTT, and protease inhibitors.

The ProteoEnrich protocol is compatible with several protein extraction reagents for mammalian cells. These reagents or kits allow for efficient extraction of functionally active proteins without secondary treatment such as sonication or freeze/thaw.

- PhosphoSafe™ Extraction Reagent (Cat. No. 71296) is an extraction reagent for mammalian and insect cells that includes four phosphatase inhibitors: sodium fluoride, sodium vanadate, β-glycerophosphate, and sodium pyrophosphate. PhosphoSafe helps maintain the phosphorylation state, and thus activation state, of both recombinant and endogenous proteins.
- CytoBuster™ Protein Extraction Reagent (Cat. No. 71009) is a proprietary formulation of detergents optimized for efficient extraction of soluble proteins from mammalian cells.
- ProteoExtract™ Subcellular Proteome Extraction Kit (S-PEK, Cat. No. 539790) is a tool for the differential extraction of proteins from adherent or suspension tissue culture cells according to their subcellular localization. The sequential extraction steps yield fractions containing (1) cytosolic proteins, (2) plasma membrane and organelle proteins, (3) soluble nuclear proteins, and (4) cytoskeletal and nuclear matrix proteins.

ATP depletion

To remove free endogenous ATP, extracts should be dialyzed or subjected to gel filtration prior to loading onto the resin. For dialysis use three buffer changes with a sample-to-volume ratio of 1:100 (e.g., for 1 ml sample use at least 100 ml dialysis buffer per buffer change). For gel filtration, use enough resin (Sephadex G-25 or equivalent) so that no more than 5% of the column volume will be loaded for buffer exchange (e.g., 20 ml column to process 1 ml of extract). Use dialysis buffer for column equilibration and elution.

Note: *In some cases, rapid removal of ATP using gel filtration instead of dialysis may yield better recovery of labile targets.*

Dialysis

1. Add PBS Tablet to 990 ml deionized water, stir or agitate until dissolved.
2. Add 10 ml 100 mM EDTA to the PBS.

Note: If 1 L of dialysis buffer is not required to process experimental sample(s) the PBS + EDTA can be stored at 4°C for up to 3 months.

3. Prepare dialysis buffer by adding 1 M DTT to PBS + EDTA solution to a final concentration of 1 mM DTT (e.g., add 100 µl 1M DTT to 99.9 ml PBS + EDTA).

Note: Prepare only enough dialysis buffer for each dialysis step, adding DTT immediately before use.

4. Dialyze extract against 100 vol dialysis buffer at 4°C for at least 4–6 h. A 3500 molecular weight cut-off is sufficient for removal of free endogenous ATP.
5. Repeat dialysis step two more times using fresh DTT added from the 1 M DTT solution.

Additives prior to binding

Before exposure of the ATP-depleted extract to the ATP-Binders™ Resin it is necessary supplement the extract with several additions, including 5X Bind Buffer, 10X Nucleotide Mix, DTT and Protease Inhibitor Cocktail.

Prior to use, the lyophilized Protease Inhibitor Cocktail Set V should be resuspended in 1 ml sterile water to make a 100X stock. Use immediately or freeze in aliquots at –20°C for long-term storage.

1. Add 1 vol ATP-Binders 5X Bind Buffer to 4 vol ATP-depleted sample.
2. Add 1 vol ATP-Binders 10X Nucleotide Mix to 10 vol sample mixture from Step 1.

Note: For samples including tyrosine kinases that require Mn^{2+} for ATP binding, add Mn^{2+} to a final concentration of 2–5 mM.

3. Add 10 µl 100 mM DTT per milliliter of sample mixture.
4. Add 10 µl resuspended Protease Inhibitor Cocktail Set V per milliliter of sample mixture.

Purification

The following protocols are for use with 50 µl swelled resin. For larger amounts of resin, increase the amount of buffers proportionately.

Buffer preparation

1. Prepare 5 ml 1X Wash Buffer by combining the following in order:

   1 ml ATP-Binders Wash Buffer Concentrate
   3.4 ml deionized water
   0.5 ml ATP-Binders 10X Nucleotide Mix
   5 µl Activated Sodium Vanadate
   Mix thoroughly, then add:
   50 µl 100 mM DTT
   50 µl Protease Inhibitor Cocktail Set V

   Mix thoroughly and keep on ice.

2. Prepare 500 µl 1X Elution Buffer by combining the following in order:

   100 µl ATP-Binders Elution Buffer Concentrate
   350 µl deionized water
   50 µl ATP-Binders 10X Nucleotide Mix
   0.5 µl Activated Sodium Vanadate
   Mix thoroughly, then add:
   5 µl 100 mM DTT
   5 µl Protease Inhibitor Cocktail Set V
   Mix thoroughly and keep on ice.

Resin pretreatment

1. Weigh 15 mg of ATP-Binders™ Resin and place in a microcentrifuge tube. The resin will swell to 50 µl.
2. Add 1 ml deionized water and 200 µl ATP-Binders 6X Resin Conditioning Buffer to the resin. Mix by vortexing for 30 s.
3. Incubate at room temperature for 30 min or at 4°C overnight.
4. Spin resin at 5,000 × g for 2 min. Decant buffer from packed resin.
5. Wash the resin 2 times by resuspending resin in 1.5 ml deionized water and mix by vortexing. Spin as in Step 4.

Resin equilibration

1. Add 1.5 ml 1X Wash Buffer to packed resin.
2. Mix by vortexing and let stand several minutes.
3. Spin at 5,000 × g for 2 min. Decant buffer from packed resin.
4. Keep tube with wet resin capped to avoid drying.

Binding, washing, and elution

1. Add ATP-depleted sample to the equilibrated ATP-Binders Resin. Incubate at 4°C for 2–3 h with gentle orbital rotation.
2. Transfer the resin/sample mixture to the sample cup of a Spin Filter. Spin at 5,000 × g for 2 min.
3. Transfer the flow-through to a fresh tube and save for analysis later.
4. Add 1 ml ice-cold 1X Wash Buffer to the resin. Close the cap of the Spin Filter and mix by vortexing. Incubate for 2 min with gentle orbital rotation.

Tip: *If using larger quantities of resin, the resin can be washed batch-wise in a microcentrifuge tube.*

5. Spin at 5,000 × g for 2 min. Remove the 1X Wash Buffer from the receiver tube.

Tip: *The wash fractions can be pooled in a separate tube for later analysis.*

6. Repeat Steps 4 and 5 twice more to complete washing the resin.

Note: *Excessive washing is not recommended because reduced recovery of certain target proteins may result.*

7. Add 150 µl ice-cold 1X Elution Buffer (2–3 resin vol) to the packed resin. Close the cap of the Spin Filter and incubate for 20 min with gentle orbital rotation.
8. Spin at 5,000 × g for 2 min.
9. Repeat Steps 7 and 8 two more times.
10. Store the elution fractions appropriately until further use.

Note: *Eluates can be used immediately or frozen at –20°C or –80°C until needed. Store eluates at a temperature compatible with target protein activity; some target proteins may be inactivated by freeze-thaw cycles.*

Sample Preparation for SDS-PAGE

To perform SDS-PAGE analysis, eluted proteins should be concentrated and excess ATP and buffer salts removed. Various physical methods can be used to accomplish this, including diafiltration and column-based buffer exchange. The following protocol is suitable for most proteins and gives a salt-free protein preparation ready for SDS-PAGE analysis.

1. Add 4 vol ice-cold methanol to 1 vol eluate.
2. Mix. Incubate on ice for 2 min.
3. Spin at 3,000 × g for 30 s.
4. Do NOT remove supernatant. Add 2 eluate vol chloroform. Mix by vortexing.
5. Add 3 eluate vol ice-cold deionized water. Vortex for 1 min.
6. Spin sample at 3,000 × g for 2 min.
7. Remove the top aqueous layer (containing salts and ATP) by pipet. Do not remove precipitated protein from the chloroform interface.
8. Add 3 eluate vol methanol. Mix by vortexing.
9. Spin sample at 3,000 × g for 2 min.
10. Remove the supernatant. Do not disturb the protein pellet.
11. Wash pellet in 1 ml methanol and mix by vortexing.
12. Spin sample at 3,000 × g for 2 min. Remove as much of the methanol as possible without disturbing the protein pellet. Allow the pellet to dry at room temperature for 15–30 min. Store at –20°C until SDS-PAGE analysis.
13. Typically, 100 µl packed ATP-Binders Resin will bind 15–25 µg protein, hence it is recommended to resuspend the pellet in no more than 25 µl 2X SDS Sample Buffer (150 mM DTT, 125 mM Tris-HCl, 15% glycerol, 3% SDS, 0.01% bromophenol blue) for a final concentration of approximately 1 mg/ml. This concentration is suitable for SDS-PAGE or Western analysis.

Reference

1. Bartnicki, D., Batenjany, M., Loomis, K., Wong, S., Menezes, R., Suleman, A., and Andrecht, S. (2004) *in**Nova**tions* **19**, 6–9.

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