Data Sheet

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ATP Affinity Test Kit

Screening Kit for the purification of ATP-binding proteins

Cat.-No.	Amount
AK-102	1 Kit (sufficient for 5 screening experiments)

Kit Contents

250 µl Aminophenyl-ATP-Sepharose®, C_{10}-linked

250 µl 8-[(6-Amino)hexyl]-amino-ATP-Sepharose®

250 µl N^6-(6-Amino)hexyl-ATP -Sepharose®

250 µl 2'/3'-EDA-ATP -Sepharose®

(each ATP-Sepharose® is pre-swollen in 20% ethanol)

10 PBS Tablets

100 µl 200 mM Sodium Orthovanadate, activated, pH 10.0

0.5 ml 100x Protease Inhibitor Mix

15 ml 5x Binding Buffer
(contains Hepes, NaCl, $MgCl_2$, and 0.25% NP-40*)

10 ml 5x Wash Buffer
(contains Hepes, NaCl, $MgCl_2$, and 0.25% NP-40*)

2 ml 5x Elution Buffer
(contains Hepes, ATP, and 0.25% NP-40*)

Additional Reagents and Material required

Microcentrifuge vials, Microcentrifuge,
H_2O, 500 mM EDTA, 100 mM DTT, 1 M DTT

Storage and Stability

Store ATP-Sepharose®, Binding Buffer and Wash Buffer at 4°C. Store Protease-Inhibitor-Mix, Elution Buffer, and Sodium Orthovanadate at -20°C. PBS tablets can be stored at room temperature. Quality guaranteed for 12 month.

Introduction

A characteristic of many proteins is their ability to bind specific small molecules (ligands) non-covalently with high affinity. This protein-ligand interaction can be used for rapid purification of a protein by affinity chromatography. In this technique, a ligand is immobilized onto the surface of a matrix (e.g. Sepharose) which is incubated with a protein mixture to be purified. The protein of interest will bind to its ligand whereas other contaminants will not. These contaminants can be washed off, and the protein of interest can be eluted by an excess of free ligand in the elution buffer.

A ligand commonly used for this technique is ATP (adenosine-5'-triphosphate) since a very large number of proteins such as kinases, motor proteins, and chaperones bind ATP with high affinity. There is however, a fundamental problem with using ATP in affinity chromatography: For attachment to a matrix ATP needs to be chemically modified with a linker (Fig. 1). This linker may interfere with the protein-ATP interaction and thereby reduce the binding.
This problem can usually be circumvented by attaching ATP at a different position at the adenine base, the sugar, or the phosphate moiety. Each of these linkage strategies has a characteristic effect upon protein-ATP interactions, and all have been applied to the purification of a variety of distinct proteins[1] (Fig. 1).

Fig. 1: Possible linkages of ATP to Sepharose® through modification of base, sugar, or phosphate moiety.

Kit description

The ATP Affinity Test Kit contains a set of 4 typical ATP-Sepharose® chromatography materials:

- Aminophenyl-ATP-Sepharose®, C_{10}-linked (***AP-ATP-S***)
- 8-[(6-Amino)hexyl]-amino-ATP-Sepharose® (***8AH-ATP-S***)
- N^6-(6-Amino)hexyl-ATP-Sepharose® (***6AH-ATP-S***)and
- 2'/3'-EDA-ATP-Sepharose® (***EDA-ATP-S***).

In ***6AH-ATP-S*** and ***8AH-ATP-S*** the ATP is immobilized via the adenine base but varies by the actual position of the linker (C6 and C8, respectively). ***AP-ATP-S*** and ***EDA-ATP-S*** are phosphate and sugar modified derivates, respectively (Fig. 2). With these four materials, one is able to identify the ideal

* NP-40 can interfere in some protein quantification assays.

derivative for purification of a particular protein of interest in a simple screening experiment. After its identification, larger quantities of the suitable ATP-Sepharose® are available for large scale protein purification (www.jenabioscience.com).

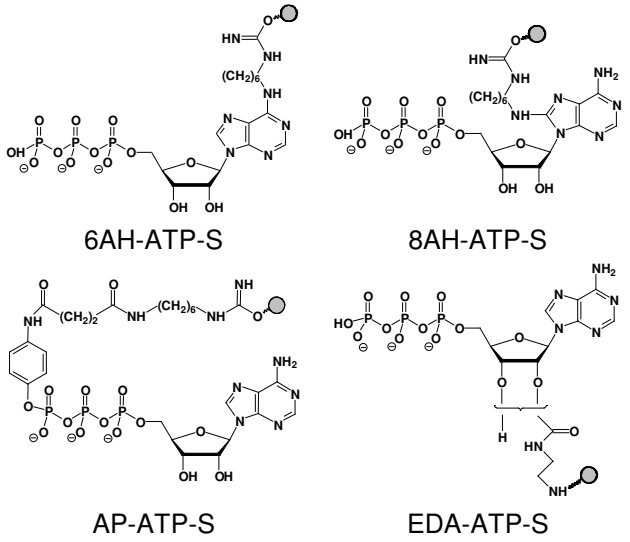

Fig. 2: Structures of the four ATP- Sepharose® materials

Example

The ATP-Sepharoses® (Fig. 2) were used to recover ATP-binding proteins from crude cell lysates from K562 (Fig. 3). Each material shows a characteristic pattern of eluted proteins: Whereas the sugar-modified ***EDA-ATP-S*** shows no major protein band (lane 5), the adenine-modified ***6AH-ATP-S and 8AH-ATP-S*** (lane 3 and 4) yield a similar pattern with a common signal at approx. 32 kDa. Phosphate-immobilized ***AP-ATP-S*** (lane 2) yields 4 major bands with a very strong signal at approx. 80 kDa.

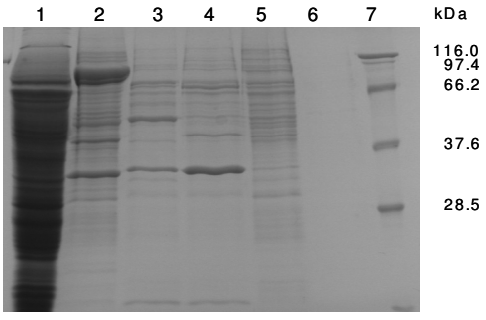

Fig. 3: 1 ml of crude cell lysate from K562 cells was incubated with 50 µl of each ATP-Sepharose® (Fig. 2) and eluates analyzed by SDS-PAGE. Lanes: **1**, crude soluble cell lysate; **2**, AP-ATP-S; **3**, 6AH-ATP-S; **4**, 8AH-ATP-S; **5**, EDA-ATP-S; **6**, Sepharose® (negative control); **7**, Molecular Weight Marker.

Properties of ATP- Sepharoses®

Table 1: Properties of ***AP-ATP-S***, ***6AH-AP-S***, ***8AH-ATP-S*** and ***EDA-ATP-S***

Bead size	45 - 165 µm
Degree of substitution	20 µmol AP-ATP/ml Sepharose® and 5 µmol/ml Sepharose® for 6AH-ATP, 8AH-ATP and EDA-ATP-S
pH stability (short term)	4 - 9
pH stability (long term)	7.5
Chemical stability	Stable to all solutions commonly used in gel filtration including 8 M urea and 6 M guanidine hydrochloride. Not stable in organic solvents!

Experimental Protocol

Sample preparation

Prepare crude protein solution using appropriate buffer conditions to maintain native protein structures and functionally active proteins. All ATP-Sepharoses® are compatible with detergents. Take an aliquot of the protein solution for later analysis.

ATP depletion

For a successful purification it is necessary to remove free endogenous ATP from the protein solution by dialysis or gel filtration before loading onto ATP-Sepharose®. For dialysis 3 buffer changes are recommended with a sample-to volume ratio of 1:100.

Dialysis

1. Add 1 PBS Tablet to 500 ml of de-ionized water and stir until dissolved.
2. Add 1 ml of 500 mM EDTA and 0.5 ml of 1 M DTT (final concentration of 1 mM each).
3. Dialyse the protein solution at 4°C for 4-6 h.
4. Exchange buffer two times.

Sample preparation

Before incubation of the ATP-depleted solution with ATP-Sepharose® add

1. 1 volume of 5x Binding Buffer per 4 volumes of the dialysed solution.

2. 10 µl of 100 mM DTT per ml of the protein solution from step 1.
3. 10 µl of 100x Protease-Inhibitor-Mix per ml of the protein solution from step 2.

Protein Purification

The following protocols are optimized for the use of 50 µl ATP-Sepharose®. For larger or smaller amounts, please increase or decrease the amount of buffers accordingly.

Buffer preparation

For 5 ml 1x Wash Buffer please add in the following order:

1 ml	5x Wash Buffer
3.9 ml	deionized water
5 µl	200 mM Sodium Orthovanadate
50 µl	100 mM DTT
50 µl	100x Protease Inhibitor Mix

For 500 µl 1x Elution Buffer please add in the following order:

100 µl	5x Elution Buffer
400 µl	deionized water
0.5 µl	200 mM Sodium orthovanadate
5 µl	100 mM DTT
5 µl	100x Protease Inhibitor Mix

Mix and keep on ice!

Equilibration of the ATP- Sepharose®

1. Add 500 µl of 1x Wash Buffer to 50 µl of ATP-Sepharose®.
2. Mix by vortexing and spin at 1000 x g for 1 min.
3. Remove Buffer and discard.
4. Repeat step 1-3 two more times.

Binding, Washing, and Elution

1. Add dialyzed protein solution to the equilibrated ATP- Sepharose®.
2. Incubate at 4°C for 2-3 h by slight agitation.
3. Spin at 1000 x g for 1 min. Transfer the supernatant to a fresh tube for later analysis.
4. Resuspend pellet in 1 ml ice-cold 1x Wash Buffer and spin at 1000 x g for 1 min. Transfer the supernatant to a fresh tube for later analysis. Wash two more times.
5. Add 150 µl ice-cold 1x Elution Buffer (2-3 volumes of the Sepharose®) to the ATP- Sepharose®. And incubate at 4°C for 20 min by slight agitation.
6. Spin at 1000 x g for 1 min. Transfer the elution fraction to a fresh tube for later analysis.
7. Repeat steps 5-6 two more times.

Analyze all fractions by SDS-PAGE in order to identify the ATP-Sepharose® most suitable for purification of the protein of interest. Larger volumes of chromatography material are available from www.jenabioscience.com.

Sepharose® is a trademark of Amersham-Biosciences Inc.

Selected references:

[1] Haystead et al. (1993) Gamma-phosphate-linked ATP-Sepharose for the affinity purification of protein-kinases - rapid purification to homogeneity of sceletal-muscle mitogen-activated protein-kinase. *Eur. J. Biochem.* **214 (2):**459.

Jenö et al. (1989) Purification and Charaterization of a 40 S Ribosomal Protein S6 Kinase from Vanadate-stimulated Swiss 3T3 Cells. *J. Biol. Chem.* **264:**1293

Trayer et. al. (1974) Affinity Chromatography of Nicotinamide Nucleotide-Dependent Dehydrogenases on Immobilized Nucleotide Derivates. *Biochem. J.* **139:**609

[2] Scherer et al. (1954) Studies on the propagation in vitro of poliomyelitis viruses. IV. Viral multiplication in a stable strain of human malignant epithelial cells (strain HeLa) derived from an epidermoid carcinoma of the cervix. *J. Exp. Med.* **97**:695.

McNutt et al. (1981) Comparison of cell peripheries in the human colonic adenocarcinoma cell lines SW480 and SW620 grown in floating chamber culture, cover slip culture, athymic (nude) mice, and BALB/c mice. *Lab. Invest.* **44:**309.