V. 1.1

PAK-GST Protein Beads
(human p21 activated kinase PBD)
Cat. # PAK02

Upon arrival store at 4°C (desiccated)
See datasheet for storage after reconstitution

Material

The Rac/Cdc42 binding domain of the human p21 activated kinase 1 protein (PAK) has been overexpressed as a GST-tagged recombinant protein in a bacterial expression system and bound to colored glutathione sepharose beads. The recombinant protein (amino acids 67-150) includes the highly conserved PBD region (also referred to as the CRIB region) and sequences required for the high affinity interaction with GTP-Rac and GTP-Cdc42 proteins. The recombinant protein is tagged with GST (28 kDa) at its amino terminus and has an approximate molecular weight of 34 kDa. PAK-GST protein beads (500 µg protein per tube) are supplied as a purple lyophilized powder. One tube of PAK02 should be sufficient for approximately 25 assays.

Storage and Reconstitution

Briefly centrifuge to collect the product at the bottom of the tube. The protein bound beads should be reconstituted to 1 mg/ml by the addition of 500 µl of distilled water. When reconstituted, the protein-beads will be in the following buffer: 50 mM Tris pH 7.5, 50 mM NaCl, 2.0% (w/v) dextran, and 10% (w/v) sucrose. The protein-bead matrix will be a purple color for easy detection. For storage, the colored bead slurry should be aliquoted into experiment sized amounts, snap frozen in liquid nitrogen and stored at -70°C. Under these conditions the protein beads are stable for 6 months. We recommend 20 µl aliquots (20 µg protein) for each experimental assay (see Biological Activity Assay in this protocol). To maintain high biological activity, the protein-bead slurry should not be exposed to repeated freeze thaw cycles. The lyophilized protein beads are stable for 1 year if stored desiccated to <10% humidity at 4°C.

Purity

Protein purity is determined by scanning densitometry of Coomassie Blue stained protein on a 12% SDS polyacrylamide gel. PAK-GST protein beads were determined to be 88% pure (see Figure 1).

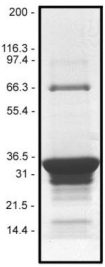

Figure 1. PAK-GST Protein Bead Purity Determination. A 20 µg sample of PAK-GST protein beads (molecular weight approx. 34 kDa) was separated by electrophoresis in a 12% SDS-PAGE system and stained with Coomassie Blue. Protein quantitation was performed using the Precision Red Protein Assay Reagent (Cat. # ADV02). Mark12 molecular weight markers are from Invitrogen.

Biological Activity Assay

PAK-GST protein specifically recognizes and binds the active "GTP-bound" forms of the Rac and Cdc42 proteins (1). It has a much lower affinity for the inactive "GDP-bound" forms of Rac and Cdc42. When coupled to a colored glutathione sepharose matrix, the PAK-GST protein beads become a convenient tool for assaying the activity of the Rac and Cdc42 proteins. A standard biological assay for PAK-GST protein beads consists of a Rac protein pulldown from human platelet extracts loaded with either GTPγS or GDP.

Reagents

1. PAK-GST protein beads (Cat. # PAK02)
2. Loading buffer (150 mM EDTA)
3. Stop buffer (600 mM $MgCl_2$)
4. Wash buffer (25 mM Tris pH 7.5, 30 mM $MgCl_2$, 40 mM NaCl)
5. Cell lysis buffer (50 mM Tris pH 7.5, 10 mM $MgCl_2$, 0.3 M NaCl, 2% IGEPAL)
6. GTPγS (20 mM solution)
7. GDP (100 mM solution)
8. Bovine or porcine brain extract (20 mg/ml) prepared in 50 mM PIPES pH 7.0,130 mM NaCl, 1 mM PMSF, 1 mM DTT, 5 µg/ml leupeptin, 0.5% Triton X-100
9. Protease inhibitor cocktail (Cat. # PIC02)
10. Anti-Rac1 monoclonal antibody (Cat. # ARC03)

Equipment

1. Microfuge at 4°C
2. SDS-PAGE and Western blot apparatus

Method

1. Dilute the brain extract to 0.5 mg/ml with cell lysis buffer containing protease inhibitors.
2. Centrifuge the extract at 16,000 x g at 4°C for 5 min to pellet cell membranes and insoluble material.
3. Aliquot 120 µg (600 µl) of the extract into two experimental tubes.
4. Add 1/10th the volume of loading buffer to each tube (final conc. 15 mM EDTA).
5. Add 1/100th the volume of GDP to one tube (final conc. 1.0 mM GDP).
6. Add 1/100th the volume of GTPγS to the other tube (final conc. 0.2 mM GTPγS).
7. Incubate both tubes at room temperature for 15 min.
8. Stop the reaction by adding 1/10th the volume of stop buffer to each tube (final conc. 60 mM $MgCl_2$).
9. Resuspend PAK-GST protein beads and add 20 µg (20 µl) protein bound beads to each reaction tube.

Phone: (303) 322.2254 **Fax:** (303) 322.2257
Customer Service: cserve@cytoskeleton.com
Technical Support: tservice@cytoskeleton.com

10. Gently rotate the tubes at 4°C for 1 h.
11. Centrifuge the beads at 5,200 x g at 4°C for 1 min.
12. Remove the supernatant and wash the beads in 500 µl of wash buffer. Repeat the wash once more to reduce background protein levels.
13. Pellet the beads and resuspend in 25 µl of SDS sample buffer.
14. The protein samples can now be analyzed by Western blot procedure using a Rac1 monoclonal antibody (Cat. # ARC03).
15. Typical assay results are shown in Figure 2.

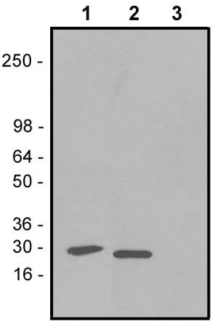

Figure 2. Selective Binding of PAK -GST Protein Beads to the GTP-bound Form of Rac1 *In Vitro*. Brain extract (120 µg) was loaded with either GTPγS (lane 2), or GDP (lane 3) as described in the method. The extracts were then incubated with 20 µg of PAK-GST protein beads. The protein-bead complexes were recovered by centrifugation and subjected to Western blot analysis using a Rac1 specific monoclonal antibody. Lane 1 shows 50 ng of recombinant His-Rac1 control protein (Note: His-Rac1 runs slightly higher than endogenous Rac due to the presence of the 6x-His tag). SeeBlue molecular weight markers are from Invitrogen

Product Uses

- Measurement of the GTP/GDP ratio of Rac1 or Cdc42 *in vitro*.
- Quantitation of GTP-Rac1/Cdc42 from tissue and tissue culture cell lysates.

References

1. Manser, E. et al. 1994. Nature. 367: 40-46.

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