V.1.0

GGA3-PBD Beads: Arf1/Arf6-GTP Affinity Beads

(*Golgi-localized γ-ear containing, Arf-binding protein 3* PBD)

Cat. # GGA07-Beads

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

Background Information

The Arf GTPases have been grouped into three classes based on their size and amino acid similarity (1): class I (Arf1 and Arf3), class II (Arf4 and Arf5) and class III (Arf6). Arf GTPases function in the secretory and endocytic pathways where they function by activating lipid modifying enzymes and recruiting proteins needed to promote coated vesicle development, scission and transport (2).

The Arf protein binding domain (PBD) of GGA3 (Golgi-localized γ-ear containing, Arf-binding protein 3) has been shown to specifically bind to GTP-bound Arf1 and Arf6 and can be used for the affinity precipitation of these active Arf isoforms (3-5).

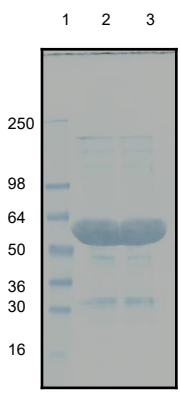
Material

The GGA3-PBD containing residues 1-316 of GGA3 has been overexpressed in a bacterial expression system. The recombinant protein has a molecular weight of approximately 60kDa and contains GST-tag at its amino terminus. The purified protein has been covalently conjugated to colored sepharose beads. Supplied as a lyophilized purple powder. One tube of GGA07 should be sufficient for approximately 20-25 assays (see Biologcial Activity Assay in this protocol).

The GGA3-PBD protein used for conjugation to the beads was analysed by scanning densitometry of a Coomassie Blue stained 4-20% SDS polyacrylamide gel and determined to be >90% pure (see Figure 1)

Figure 1. GGA3-PBD Protein Purity Determination

A 20 µg sample of GGA3-PBD protein from two different Lots; Lanes 2 & 3 (molecular weight approx. 60 kDa) was separated by electrophoresis in a 4-20% SDS polyacrylamide gel, 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.



Storage and Reconstitution

Shipped at ambient temperature. The recommended storage conditions for the lyophilized material is 4°C and <10% humidity. Under these conditions the protein is stable for 1 year.

Briefly centrifuge to collect the protein 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. The protein will be in the following buffer; 25 mM Tris pH 7.5, 25 mM NaCl, 5% (w/v) sucrose and 1% (w/v) dextran. 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. The protein is stable for 6 months under these conditions. 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 must not be exposed to repeated freeze-thaw cycles.**

Biological Activity Assay

The GGA3-PBD protein specifically recognizes and binds the active "GTP-bound" forms of the Arf1 and Arf6 proteins (3). It has a much lower affinity for the inactive "GDP-bound" forms of Arf1 and Arf6. When coupled to a colored sepharose matrix, the GGA3-PBD beads become a convenient tool for assaying the activity of Arf1 and Arf6 proteins. A standard biological assay for GGA3-PBD beads consists of a Arf1 protein pull-down from *in vitro* activated Arf1 (Method #1) or *in vivo* activated Arf1 (Method #2).

Method #1: Pull-Down Assay with In Vitro Loaded Lysates

1) Grow one 150 cm2 plate of MDCK cells to 70% confluency in EMEM media plus 10% fetal bovine serum (FBS). Cells should be grown in a tissue culture incubator set at 5% CO_2/37°C/95% humidity. NOTE: any cell line containing Arf1 can be used for this assay.
2) Remove cells from incubator and wash with ice cold PBS pH7.4.
3) Harvest cells with 1ml of Cell Lysis Buffer (50 mM Tris pH 7.5, 10 mM $MgCl_2$, 0.3M NaCl, 2% Igepal, 0.01% Sodium dodecyl sulfate).
4) Dilute the cell lysates to 1 mg/ml with Cell Lysis Buffer.
5) Aliquot 500 µg (500 µl) of the extract into two experimental tubes.
6) Add 1/15th the volume of loading buffer (150 mM EDTA) to each tube (final conc. 10 mM EDTA).
7) Add 1/100th the volume of 100 mM GDP to one tube (final conc. 1 mM GDP).
8) Add 1/100th the volume of 20 mM GTPγS to the other tube (final conc. 0.2 mM GTPγS).
9) Incubate both tubes at 37°C for 20 min.
10) Stop the reaction by adding 1/10th the volume of stop buffer (600 mM $MgCl_2$) to each tube (final conc. 60 mM $MgCl_2$).

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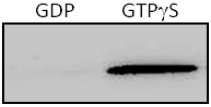
11) Resuspend GGA07 beads to 1 mg/ml by the addition of 500 µl distilled water as per storage and reconstitution section and add 20 µg (20 µl) to each reaction tube.
12) Gently rotate the tubes at 4°C for 1h.
13) Pellet the beads at 8k rpm in a microfuge at 4°C for 1 min.
14) Remove the supernatant and wash the beads in 500 µl of Wash buffer. Repeat the wash once more to reduce background protein levels.
15) Pellet the beads as before and resuspend in 20 µl of SDS sample buffer.
16) The protein samples can now be analyzed by Western blot using an Arf1 or Arf6 specific monoclonal antibody.
17) Typical assay results are shown in Figure 2.

Figure 2. ***Selective Binding of GGA07 Beads to the GTPγS-bound Form of Arf1 in vitro***

MDCK cell lysates (500 µg) were loaded with either GTPγS or GDP, subjected to a pull-down assay with 20 µg of GGA3-PBD beads and analyzed by Western blot using a Arf1 specific monoclonal antibody at 1:250 dilution.

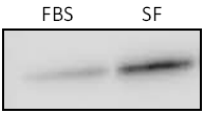


Method #2: Activation of Arf1 in C2C12 Cells by Serum Starvation

1) Grow two 150 cm^2 plates of C2C12 cells (ATCC Cat# CRL-1772) to approximately 50% confluency in DMEM media supplemented with 10% fetal bovine serum (FBS). Cells should be grown in a tissue culture incubator set at 5% CO_2/37°C/95% humidity.
2) Passage the cells once into eight 150 cm^2 plates and grow as above to 30% confluency.
3) Wash cells twice with serum free DMEM.
4) Replace DMEM/FBS media into four plates (FBS plates) and DMEM only into the remaining four plate (SF plates).
5) Return cells to the tissue culture incubator for 1h.
6) Remove cells from incubator and wash with ice cold PBS pH7.4.
7) Harvest cells with 200 µl each of Cell Lysis Buffer (50 mM Tris pH 7.5, 10 mM $MgCl_2$, 0.3M NaCl, 2% Igepal, 0.01% Sodium dodecyl sulfate) and pool the FBS plate lysates into one tube and the SF plate lysates into a second tube.
8) Resuspend GGA07 beads to 1 mg/ml by the addition of 500 µl distilled water as per storage and reconstitution section and add 20 µg (20 µl) to each reaction tube.
9) Gently rotate the tubes at 4°C for 1h.
10) Pellet the beads at 8k rpm in a microfuge at 4°C for 1 min.
11) Remove the supernatant and wash the beads in 500 µl of Wash buffer. Repeat the wash once more to reduce background protein levels.
12) Pellet the beads as before and resuspend in 20 µl of SDS sample buffer.
13) The protein samples can now be analyzed by Western blot using an Arf1 specific monoclonal antibody (Cat. # ARF01) at 1:250 dilution.
14) Typical assay results are shown in Figure 3.

Figure 3. ***Selective Binding of GGA07 Beads to the GTP-bound Form of Arf1 C2C12 lysates***

C2C12 cell lysates (500 µg) from untreated cells (FBS) or cells that were serum starved for 1h (SF) were assayed as described in Method #2. Active (GTP-bound) Arf1 was detected by western blot analysis using an anti-Arf1 antibody as described in the method.



Product Uses

- Measurement of the GTP/GDP ratio of Arf1 and Arf6 *in vitro*.
- Quantitation of GTP-Arf1/6 from tissue and tissue culture lysates.

References

1. Tsuchiya, M., Price, S. R., Tsai, S-C, Moss, J. and Vaughan, M. (1991) Molecular Identification of ADP-Ribosylation Factor mRNAs and Their Expression in Mammalian Cells. *J. Biol. Chem.* **266**, 2772-2777.
2. D'Souza-Schorey, C., and Chavrier, P. (2006) ARF proteins: Roles in membrane traffic and beyond. *Nat. Rev. Mol. Cell Biol.* **7**, 347-358.
3. Takatsu, H., Yoshino, K., Toda, K., and Nakayama, K. (2002) GGA proteins associate with Golgi membranes through interaction between their GGAH domains and ADP-ribosylation factors. Biochem. J. **365**, 369-378.
4. Yoon, HY, Bonifacino, J. S., and Randazzo, P. A. (2005) In Vitro Assays of Arf1 Interaction with GGA Proteins. Methods Enzymol. **404**, 316-332.
5. Cohen, L. A., and Donaldson, J. G. (2010) Analysis of Arf GTP-binding protein function in cells. Curr. Protoc. Cell Biol. **48**, 14.12.1-14.12.17.

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