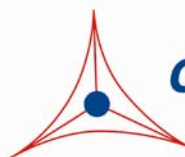

Product Manual

Arf6 Activation Assay Kit, Trial Size

Catalog Number

STA- 407- 6- T 5 assays

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures



CELL BIOLABS, INC.

Creating Solutions for Life Science Research

Introduction

Small GTP-binding proteins (or GTPases) are a family of proteins that serve as molecular regulators in signaling transduction pathways. Arf, a 20 kDa protein of the Ras superfamily, regulates a variety of biological response pathways that include vesicle trafficking, organelle structure, and endocytosis. Like other small GTPases, Arf regulates molecular events by cycling between an inactive GDP-bound form and an active GTP-bound form. In their active (GTP-bound) state, Arf1 and Arf6 bind specifically to the protein-binding domain (PBD) of GGA3 to control downstream signaling cascades.

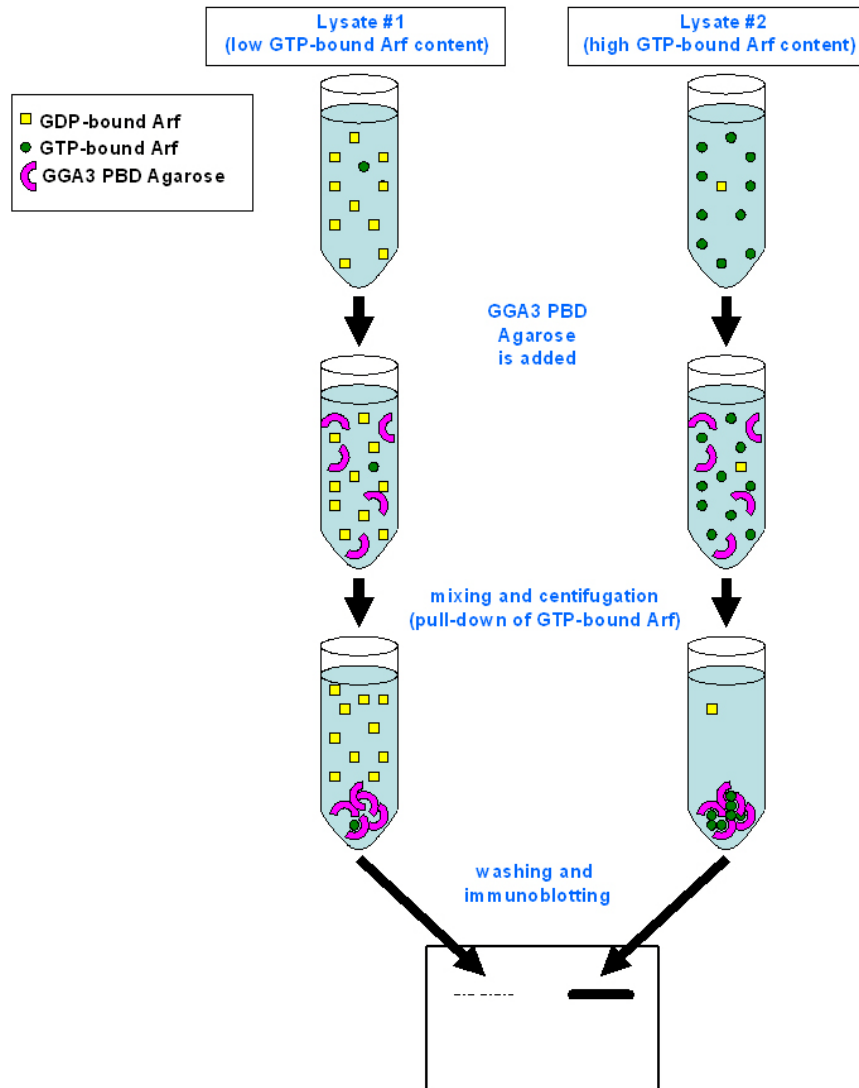
Cell Biolabs' Arf6 Activation Assay Kit utilizes GGA3 PBD Agarose beads to selectively isolate and pull-down the active form of Arf from purified samples or endogenous lysates. Subsequently, the precipitated GTP-Arf is detected by western blot analysis using an anti-Arf6 antibody.

Cell Biolabs' Arf6 Activation Assay Kit provides a simple and fast tool to monitor the activation of Arf. The kit includes easily identifiable GGA3 PBD Agarose beads (see Figure 1), pink in color, and a GTPase Immunoblot Positive Control for quick Arf identification. This Trial Size kit provides sufficient quantities to perform 5 assays.



Figure 1: GGA3 PBD Agarose beads, in color, are easy to visualize, minimizing potential loss during washes and aspirations.

Assay Principle



Related Products

1. STA-400: Pan-Ras Activation Assay Kit
2. STA-400-H: H-Ras Activation Assay Kit
3. STA-400-K: K-Ras Activation Assay Kit
4. STA-400-N: N-Ras Activation Assay Kit
5. STA-401-1: Rac1 Activation Assay
6. STA-401-2: Rac2 Activation Assay
7. STA-403-A: RhoA Activation Assay
8. STA-403-B: RhoB Activation Assay

9. STA-403-C: RhoC Activation Assay
10. STA-404: Rac1/Cdc42 Activation Assay Combo Kit
11. STA-407-1: Arf1 Activation Assay
12. STA-410: Raf1 RBD Agarose Beads
13. STA-457: Ras Expression Vector Set
14. STA-459: Active Ras Expression Vector Set

Kit Components

1. GGA3 PBD Agarose (Part No 240701-T): One 200 μ L vial of 50% slurry, 100 μ g human GGA3 PBD (amino acid 1-316) in PBS containing 50% glycerol.
Note: Agarose bead appears pink in color for easy identification, washing, and aspiration.
2. 100X GTP γ S (Part No. 240103-T): One 20 μ L vial of 10 mM GTP γ S dissolved in sterile water.
3. 100X GDP (Part No. 240104-T): One 20 μ L vial of 100 mM GDP dissolved in sterile water.
4. 5X Assay/Lysis Buffer (Part No. 240102-T): Four 2 mL vials of 125 mM HEPES, pH 7.5, 750 mM NaCl, 5% NP-40, 50 mM MgCl₂, 5 mM EDTA, 10% Glycerol.
5. Anti-Arf6, Mouse Monoclonal (Part No. 240703-T): One 10 μ L vial in PBS, pH 7.4, 0.05% NaN₃, 0.1% BSA. The antibody reacts with Arf6 from human, bovine, canine, and rat.
6. GTPase Immunoblot Positive Control (Part No. 240105): One 100 μ L vial of 293 cell lysate at 0.5 mg/mL (provided ready-to-use in 1X reducing SDS-PAGE Sample Buffer, pre-boiled)

Materials Not Supplied

1. Stimulated and non-stimulated cell lysates
2. Arf activators
3. Protease inhibitors
4. 0.5 M EDTA in water
5. 1 M MgCl₂
6. 30°C incubator or water bath
7. 4°C tube rocker or shaker
8. 2X reducing SDS-PAGE sample buffer
9. Electrophoresis and immunoblotting systems
10. Immunoblotting wash buffer such as TBST (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.05% Tween-20)
11. Immunoblotting blocking buffer (TBST containing 5% Non-fat Dry Milk)
12. PVDF or nitrocellulose membrane
13. Secondary Antibody
14. ECL Detection Reagents

Storage

Store all kit components at -20°C. The 5X Assay/Lysis Buffer may be stored at either -20°C or 4°C. Avoid multiple freeze/thaw cycles.

Preparation of Reagents

- 1X Assay/Lysis Buffer: Mix the 5X Stock briefly and dilute to 1X in deionized water. Just prior to usage, add protease inhibitors such as 1 mM PMSF, 10 µg/mL leupeptin, and 10 µg/mL aprotinin.

Preparation of Samples

Note: It is advisable to use fresh cell lysates because GTP-Arf is quickly hydrolyzed to GDP-Arf; frozen lysates stored at -70°C may be used. Performing steps at 4°C or on ice may reduce hydrolysis. Avoid multiple freeze/thaw cycles of lysates.

I. Adherent Cells

1. Culture cells to approximately 80-90% confluence. Stimulate cells with Arf activator(s) as desired.
2. Aspirate the culture media and wash twice with ice-cold PBS.
3. Completely remove the final PBS wash and add ice-cold 1X Assay/Lysis Buffer to the cells (0.5 - 1 mL per 100 mm tissue culture plate).
4. Place the culture plates on ice for 10-20 minutes.
5. Detach the cells from the plates by scraping with a cell scraper.
6. Transfer the lysates to appropriate size tubes and place on ice.
7. If nuclear lysis occurs, the cell lysates may become very viscous and difficult to pipette. If this occurs, lysates can be passed through a 27½-gauge syringe needle 3-4 times to shear the genomic DNA.
8. Clear the lysates by centrifugation for 10 minutes (14,000 x g at 4°C).
9. Collect the supernatant and store samples on ice for immediate use, or snap freeze and store at -70°C for future use.
10. Proceed to GTPγS/GDP Loading for positive and negative controls, or Pull-Down Assay.

II. Suspension Cells

1. Culture cells and stimulate with Arf activator(s) as desired.
2. Perform a cell count, and then pellet the cells by centrifugation.
3. Aspirate the culture media and wash twice with ice-cold PBS.
4. Completely remove the final PBS wash and add ice-cold 1X Assay/Lysis Buffer to the cell pellet (0.5 – 1 mL per 1×10^7 cells).
5. Lyse the cells by repeated pipetting.

6. Transfer the lysates to appropriate size tubes and place on ice.
7. If nuclear lysis occurs, the cell lysates may become very viscous and difficult to pipette. If this occurs, lysates can be passed through a 27½-gauge syringe needle 3-4 times to shear the genomic DNA.
8. Clear the lysates by centrifugation for 10 minutes (14,000 x g at 4°C).
9. Collect the supernatant and store samples on ice for immediate use, or snap freeze and store at -70°C for future use.
10. Proceed to GTPγS/GDP Loading for positive and negative controls, or Pull-Down Assay.

Assay Protocol

Important Note: Before running any Small GTPase pulldown assay, it is always a good practice to run a Western Blot directly on the cell lysate using the antibody provided in this kit. For example: load 5 μg, 10 μg and 20 μg of lysate onto an SDS-PAGE gel, transfer and blot. When proceeding with the pulldown assay, use 100-times the amount of lysate that gave you a clear band of your desired small GTPase in the direct Western blot. For example: if the 5 μg band was faint but the 10 μg band was clear and strong, use 100 x 10 μg = 1 mg of lysate in the assay. Using sufficient lysate in the pulldown assay is critical to success.

I. GTPγS/GDP Loading (Positive and Negative Controls)

Note: Samples that will not be GTPγS/GDP loaded may be kept on ice during the loading of controls.

1. Aliquot 0.5 – 1 mL of each cell lysate to two microcentrifuge tubes.
Note: Typical protein content/sample is > 0.5 mg.
2. Adjust the volume of each sample to 1 mL with 1X Assay Lysis Buffer.
3. Add 20 μL of 0.5 M EDTA to each sample.
4. Add 10 μL of 100X GTPγS to one tube (positive control) and 10 μL of 100X GDP to the other tube (negative control). Mix and label each tube appropriately.
5. Incubate the tubes for 30 minutes at 30°C with agitation.
6. Stop the loading by adding 65 μL of 1 M MgCl₂ to each tube. Mix and place tubes on ice.
7. Continue with Pull-Down assay.

II. Arf Pull-Down Assay

1. Aliquot 0.5 – 1 mL of cell lysate (treated with Arf activators or untreated) to a microcentrifuge tube.
2. Adjust the volume of each sample to 1 mL with 1X Assay Lysis Buffer.
3. Thoroughly resuspend the GGA3 PBD Agarose bead slurry by vortexing or titrating.
4. Quickly add 40 μL of resuspended bead slurry to each tube (including GTPγS/GDP controls).
5. Incubate the tubes at 4°C for 1 hour with gentle agitation.
6. Pellet the beads by centrifugation for 10 seconds at 14,000 x g.

7. Aspirate and discard the supernatant, making sure not to disturb/remove the bead pellet.
8. Wash the bead 3 times with 0.5 mL of 1X Assay Buffer, centrifuging and aspirating each time.
9. After the last wash, pellet the beads and carefully remove all the supernatant.
10. Resuspend the bead pellet in 40 μ L of 2X reducing SDS-PAGE sample buffer.
11. Boil each sample for 5 minutes.
12. Centrifuge each sample for 10 seconds at 14,000 x g.

III. Electrophoresis and Transfer

1. Load 20 μ L/well of pull-down supernatant to a polyacrylamide gel. Also, it's recommended to include a pre-stained MW standard (as an indicator of a successful transfer in step 3).
Note: If desired, 10 μ L/well of GTPase Immunoblot Control (provided ready-to-use, pre-boiled) can be added as an immunoblot positive control.
2. Perform SDS-PAGE as per the manufacturer's instructions.
3. Transfer the gel proteins to a PVDF or nitrocellulose membrane as per the manufacturer's instructions.

IV. Immunoblotting and Detection (all steps are at room temperature, with agitation)

1. Following the electroblotting step, immerse the PVDF membrane in 100% Methanol for 15 seconds, and then allow it to dry at room temperature for 5 minutes.
Note: If Nitrocellulose is used instead of PVDF, this step should be skipped.
2. Block the membrane with 5% non-fat dry milk in TBST for 1 hr at room temperature with constant agitation.
Incubate the membrane with Anti-Arf6 Antibody, freshly diluted 1:200 to 1:1000 in 5% non-fat dry milk/TBST, for 1-2 hr at room temperature with constant agitation.
Note: To conserve antibody, incubations should be performed in a plastic bag.
3. Wash the blotted membrane three times with TBST, 5 minutes each time.
4. Incubate the membrane with a secondary antibody (e.g. Goat Anti-Mouse IgG, HRP-conjugate), freshly diluted in 5% non-fat dry milk/TBST, for 1 hr at room temperature with constant agitation.
5. Wash the blotted membrane three times with TBST, 5 minutes each time.
6. Use the detection method of your choice. We recommend enhanced chemiluminescence reagents from Pierce.

Example of Results

The following figure demonstrates typical results seen with Cell Biolabs Arf6 Activation Assay Kit. One should use the data below for reference only.

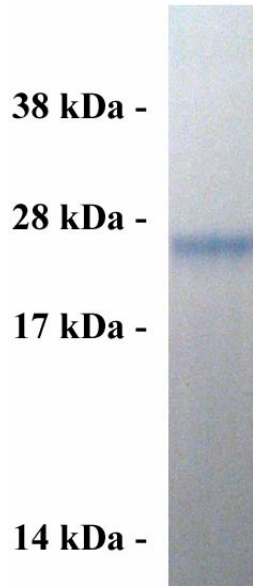


Figure 2: GTPase Immunoblot Positive Control

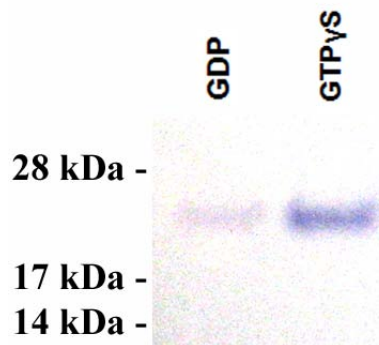


Figure 3: Arf6 Activation Assay. *Lane 1*, HeLa cell lysate loaded with GDP and incubated with GGA3 PBD Agarose beads. *Lane 2*, HeLa cell lysate loaded with GTP γ S and incubated with GGA3 PBD Agarose beads.

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Recent Product Citations

1. Matsumoto, Y. et al. (2016). Inhibition of epithelial-mesenchymal transition by cetuximab via the EGFR-GEP100-Arf6-AMAP1 pathway in head and neck cancer. *Head Neck* doi:10.1002/hed.24626.
2. Dutta, D. & Donaldson, J. G. (2015). Sorting of clathrin-independent cargo proteins depends on Rab35 delivered by clathrin-mediated endocytosis. *Traffic*. doi: 10.1111/tra.12302.
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4. Grossmann, A. et al. (2013). The Small GTPase ARF6 stimulates β -catenin transcriptional activity during WNT5A-mediated melanoma invasion and metastasis. *Sci. Signal.* 6:ra14.

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