INSTRUCTIONS

Thermo SCIENTIFIC

Active Arf1 Pull-Down and Detection Kit

16121 2272.0

Number	Description
16121	**Active Arf1 Pull-Down and Detection Kit**, contains sufficient reagents for 30 pull-down reactions

Kit Contents:

Box 16121X (items ship with dry ice)

GST-GGA3-PBD, 3mg, contains 1-2mg/mL in 25mM Tris•HCl, pH 7.2, 150mM NaCl and 10% glycerol; GST-GGA3-PBD interacts with Arf1 from human, mouse, rat, dog and possibly from all mammalian species; store at -70°C

100X GTPγS, 50µL, 10mM in sterile water; store at -70°C (or -20°C)

100X GDP, 50µL, 100mM in sterile water; store at -70°C (or -20°C)

Box 16121Y (items ship with an ice pack)

Anti-Arf1 Antibody, 25µL (5 units) rabbit monoclonal IgG; reacts with Arf1 of human, rat, mouse and dog; store at -20°C. Note: One unit is defined as the amount of antibody required to efficiently detect Arf1 in 20µg NIH3T3 whole cell lysate by Western blotting (8.5 × 7.5cm membrane).

Glutathione Resin, 3mL, supplied as 50% slurry containing 0.05% sodium azide; store at 4°C

1X Lysis/Binding/Wash Buffer, 100mL, contains 25mM Tris•HCl, pH 7.2, 150mM NaCl, 5mM $MgCl_2$, 1% NP-40, and 5% glycerol, store at 4°C

2X SDS Sample Buffer, 1.5mL, contains 125mM Tris•HCl, pH 6.8, 2% glycerol, 4% SDS (w/v) and 0.05% bromophenol blue; store at 4°C

Spin Cups, 30 each, maximum volume 850µL, store at room temperature or 4°C

Collection Tubes, 90 each, store at room temperature or 4°C

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Introduction

The Thermo Scientific Active Arf1 Pull-Down and Detection Kit is a simple and fast tool to monitor Arf1 small GTPase activation. The kit includes a GST-fusion protein of the GGA3 protein-binding domain (PBD) and glutathione resin to specifically pull-down active Arf1 and a specific antibody for Western blot detection. Also included are two control nucleotides, GTPγS and GDP, for generating positive and negative control lysates, respectively. Every kit is functionally tested to ensure component performance.

Small GTPases serve as molecular switches in signaling transduction pathways. Arf1 (~21 kDa) regulates the assembly of different coat proteins (clathrin) onto budding vesicles on the trans-golgi network and endosomal membranes. Arf1 is also important for the maintenance of organelle structure. Like other small GTPases, Arf1 is active when bound to GTP and inactive when bound to GDP.

Important Product Information

- Arf1-GTP is quickly hydrolyzed to Arf1-GDP; therefore, prepare lysate just before use for each assay.
- The Lysis/Binding/Wash buffer is compatible with the Thermo Scientific Pierce BCA and Pierce 660nm Protein Assays but not the Coomassie (Bradford) Protein Assay.
- For best results always use protease inhibitors for cell lysis and keep lysates on ice between steps.
- For optimal pilot experiments, use 500µg to 1mg of total lysate per assay.
- The anti-Arf1 antibody reacts with Arf1 from human, rat, mouse and dog.
- For best results when performing the Western blotting procedure, use Pierce® Goat Anti-Rabbit IgG (H+L), Peroxidase Conjugated (Product No. 31460) and Thermo Scientific SuperSignal West Pico Chemiluminescent Substrate (Product No. 34080). (Refer to Additional Information section, Figure 1.) If similar products from other vendors are used, the Western blotting procedure must be optimized.

Additional Materials Required

- Protease inhibitors (e.g., Thermo Scientific Halt Protease Inhibitor Single-Use Cocktail – EDTA Free, Product No. 78425)
- Pierce BCA Protein Assay Reagent (Product No. 23227) or Pierce 660nm Protein Assay (Product No. 22660)
- β-mercaptoethanol (Product No. 35602) or dithiothreitol (DTT; Product No. 20291)
- Polyacrylamide gel, 12% or 4-20% (Thermo Scientific Precise Protein Gels; see catalog or website)
- Nitrocellulose (Product No. 88014) or PVDF (Product No. 88585) membrane
- Tris-buffered saline (TBS; 25mM Tris•HCl, pH 7.5, 150mM NaCl; Product No. 28379 or 28358)
- Tween®-20 Detergent (Product No. 28320)
- Thermo Scientific StartingBlock Blocking Buffer (Product No. 37538 or 37542)
- Pierce Goat Anti-Rabbit IgG-Horseradish Peroxidase Conjugate (Product No. 31460)
- SuperSignal® West Pico Chemiluminescent Substrate (Product No. 34080)
- Thermo Scientific CL-XPosure X-ray Film (Product No. 34090 or 34091) or a CCD camera
- 0.5M EDTA, pH 8.0
- 1M $MgCl_2$
- Sodium azide (NaN_3)

Procedure for Arf1 Pull-Down and Detection

A. Cell Lysis

Note: Add protease inhibitors to Lysis/Binding/Wash Buffer before use.

- **For adherent cells:**

1. Carefully remove the culture medium and gently rinse the cells once with ice-cold TBS.
2. Add 0.5-1.0mL of Lysis/Binding/Wash Buffer per 75cm^2 flask or 0.3-0.5mL per 100mm plate with cells at 80-90% confluency.
3. Scrape the cells and transfer to a microcentrifuge tube. Vortex the tube briefly and incubate on ice for 5 minutes.
4. Centrifuge at 16,000 × *g* at 4°C for 15 minutes.
5. Transfer the supernatant (total lysate) to a new tube.

- **For non-adherent cells:**

1. Pellet cells from one 75cm^2 flask (approx. 1-2 × 10^7 cells) at 100 × *g* for 5 minutes. Resuspend cells in 10mL ice-cold TBS.
2. Pellet the cells at 100 × *g* for 5 minutes and carefully remove TBS.
3. Add 0.5-1.0ml Lysis/Binding/Wash Buffer to the cell pellet and resuspend the pellet.
4. Transfer the sample to a microcentrifuge tube and incubate on ice for 5 minutes.
5. Centrifuge at 16,000 × *g* at 4°C for 15 minutes.
6. Transfer the supernatant (total lysate) to a new tube.

B. *In vitro* GTPγS or GDP Treatment (Optional)

Experiment control treatments include GTPγS (positive control) and GDP (negative control). Use 500µg of cell lysate for each treatment. For best results, aliquot GTPγS and GDP at first use to minimize freeze/thaw cycles.

1. Add 10µL of 0.5M EDTA, pH 8.0 (for a final concentration of 10mM) to 500µL of lysate and vortex.
2. Add 5µL of 10mM GTPγS (for a final concentration of 0.1mM) or 5µL of 100mM GDP (for a final concentration of 1mM) and vortex.
3. Incubate the mixture at 30°C for 15 minutes with constant agitation.
4. To terminate the reaction, place sample on ice, add 32µL of 1M $MgCl_2$ (for a final concentration of 60mM) and vortex.

C. Affinity Precipitation of Activated Arf1

Reserve a portion of the cell lysate for protein assay using the Pierce BCA or 660nm Protein Assay.

1. Place a spin cup into a collection tube for each sample.
2. Swirl the bottle of Glutathione Resin to thoroughly resuspend the resin. Add 100µL of the 50% resin slurry to the spin cup with collection tube. Centrifuge the tube at 6,000 × *g* for 10-30 seconds and discard the flow-through.
3. Add 400µL of Lysis/Binding/Wash Buffer to each tube with resin. Invert the tubes gently several times. Centrifuge the tubes at 6,000 × *g* for 10-30 seconds and discard the flow-through.
4. Thaw the GST-GGA3-PBD on ice and immediately make 100µg aliquots. Store aliquots for later use at -70°C.
5. Add 100µg of GST-GGA3-PBD to the spin cup containing the Glutathione Resin and immediately transfer up to 700µL of the cell lysate (containing at least 500µg of total proteins) to the spin cup.
6. Close the cap and seal the collection tube with laboratory film to prevent leakage, which is caused by detergent in the lysate. Vortex the sample.
7. Incubate the reaction mixture at 4°C for 1 hour with gentle rocking.

8. Centrifuge the spin cup with collection tube at 6,000 × *g* for 10-30 seconds. Remove the laboratory film and transfer the spin cup to a new collection tube.
9. Add 400µL of Lysis/Binding/Wash Buffer, invert the tube three times and centrifuge at 6,000 × *g* for 10-30 seconds. Discard the flow-through. Repeat this wash step two additional times.
10. Transfer the spin cup to a new collection tube.
11. Prepare 50µL of reducing sample buffer for each pull-down reaction by mixing 1 part β-mercaptoethanol to 20 parts 2X SDS Sample Buffer (e.g., 2.5µL of β-mercaptoethanol to 50µL of 2X SDS Sample Buffer), or by adding dithiothreitol (DTT) to a final concentration of 200mM.
12. Add 50µL 2X reducing sample buffer to the resin. Vortex the sample and incubate at room temperature for 2 minutes.
13. Centrifuge the tube at 6,000 × *g* for 2 minutes. Remove and discard the spin cup containing the resin.
14. Heat the eluted samples for 5 minutes at 95-100°C. Separate proteins by SDS-PAGE or store sample at -20°C until use. Apply at least 25µL per lane for a 10 × 10 cm mini-gel (12% or 4-20% polyacrylamide gel provides the best separation).

D. Western Blot Analysis

This procedure has been optimized using SuperSignal West Pico Chemiluminescent Substrate (see Important Product Information section). Perform all blocking, probing and washing incubation steps using constant agitation.

1. Separate the proteins by SDS-PAGE and transfer to nitrocellulose or PVDF membrane.

   Note: Include unfractionated cell lysate as a control to verify that the Western blot analysis is functioning properly.
2. Block membrane in StartingBlock™ Blocking Buffer with 0.05% Tween-20 Detergent for 1 hour.

   Note: Either TBST- or PBST-based blocking and washing may be used. BSA (5%) can be used as an alternative to StartingBlock Blocking Buffer.
3. Prepare the Anti-Arf1 Antibody (1:2,000) in StartingBlock Blocking Buffer with 0.05% Tween-20 Detergent. Incubate the membrane in the primary anti-Arf1 antibody solution for 1 hour.

   Note: If the number of pull-down reactions per blot is low, the diluted anti-Arf1 antibody solution can be re-used up to three times with no performance loss. If using an alternative blocking buffer, add 0.1% NaN_3 for antibody storage.
4. Wash the membrane five times for 5 minutes each with TBS containing 0.05% Tween-20 Detergent (TBST).
5. Dilute the anti-rabbit IgG-HRP Conjugate in StartingBlock Blocking Buffer with 0.05% Tween-20 Detergent [e.g., if using Pierce Goat Anti-Rabbit IgG (H+L], Peroxidase Conjugated dilute within 1:20,000 to 1:100,000).
6. Incubate membrane in the anti-rabbit IgG-HRP Conjugate solution at room temperature for 1 hour.
7. Wash the membrane five times for 5 minutes each with TBST.
8. Incubate membrane with SuperSignal West Pico Chemiluminescent Substrate for 5 minutes.
9. Immediately expose the membrane to X-ray film or a CCD camera.

   Note: The Arf1 band is located at ~21kDa.

Troubleshooting

Problem	Cause	Solution
No activated Arf1 detected	Primary antibody requires optimization	Empirically determine the optimal primary antibody concentration
	Incorrect secondary antibody used for detection	Use goat anti-rabbit IgG conjugated to HRP
	No activated Arf1 present in lysates	Include GTPγS-treated lysate as a pull-down positive control
	Insufficient activated Arf1	Increase the amount of lysate used for detection
	GST-GGA3-PBD was omitted or was degraded	Avoid multiple freeze/thaw cycles of GST-GGA3-PBD
	Degraded proteins	Add protease inhibitors to the Lysis/Binding/Wash Buffer before lysing cells
	HRP substrate is not functioning properly or requires optimization	Consult the instructions for the detection system being used
No signal with GTPγS or strong signal with GDP	GTPγS or GDP are no longer functional	Aliquot GTPγS or GDP after the first thaw and store at -70°C; avoid repeated freeze/thaw cycles
	Incorrect concentration of EDTA or $MgCl_2$	Prepare new solutions with correct concentration
Western blot resulted in high background	Inadequate blocking and/or washing	Consult the instructions for the HRP substrate being used
	Secondary antibody concentration is too high	

Additional Information

Arf1 is active when bound to GTP and inactive when bound to GDP. Active Arf1 binds specifically to protein-binding domain (PBD) of GGA3, a downstream effector. Therefore, the PBD of GGA3 can be used as a probe to specifically isolate the active form of Arf1 (Figure 1).

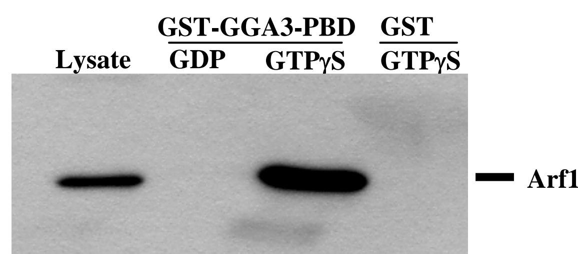

Figure 1. Western blot of control reactions. NIH3T3 cell lysates (500µg) were treated *in vitro* with GTPγS or GDP to activate or inactivate Arf1 (refer to Section B). The lysates were incubated with GST-GGA3-PBD and Glutathione Resin. GTPγS-treated lysate was also incubated with GST alone in the presence of a Glutathione Resin (negative control). Fifty percent of the eluted samples (25µL) and 10µg of cell lysate were separated by 4-20% SDS-PAGE, transferred to a PVDF membrane and probed with Anti-Arf1 antibody. Pierce Goat Anti-Rabbit IgG (H+L), Peroxidase Conjugated (Product No. 31460; 1:20,000 dilution) was used as the secondary antibody. The detection was achieved with SuperSignal West Pico Chemiluminescent Substrate (Product No. 34080) and followed by exposure to X-ray film.

Related Thermo Scientific Products

25200-25244	**Precise™ Protein Gels** (see catalog or web site for a complete listing)
21065	**Pierce Background Eliminator Kit,** for eliminating background from overexposed X-ray film
23236	**Pierce Coomassie Plus (Bradford) Protein Assay Reagent**
23227	**BCA Protein Assay Reagent Kit**
22660	**Pierce 660nm Protein Assay Reagent,** 750mL
28320	**Surfact-Amps® 20 (10% Tween-20 Detergent)**, 6 × 10mL
28379	**BupH™ Tris Buffered Saline Packs,** 10 packs, each makes 500mL
28372	**BupH Phosphate Buffered Saline Packs,** 40 packs, each makes 500mL
28358	**Tris Buffered Saline, 20X**, 500mL
28348	**Phosphate Buffered Saline, 20X**, 500mL
37538	**StartingBlock (PBS) Blocking Buffer**, 1L
37542	**StartingBlock (TBS) Blocking Buffer**, 1L
78425	**Halt™ Protease Inhibitor Single-Use Cocktail, EDTA-free (100X),** 24 × 100µL microtubes
31460	**Pierce Goat Anti-Rabbit IgG (H+L), Peroxidase Conjugated**, 2mL
34079	**SuperSignal West Pico Chemiluminescent Substrate**, 500mL
34090	**CL-XPosure™ Film (5" × 7")**, 100 sheets/pkg
20291	**Dithiothreitol (DTT), No-Weigh™,** 7.7mg × 48 microtubes
88014	**Nitrocellulose Membrane**, 0.45µm, 7.9cm × 10.5cm
88585	**PVDF Membrane,** 0.45µm, 7.9cm × 10.5cm
46430	**Restore® Plus Western Blot Stripping Buffer,** 500mL
20237	**GST,** 1mg
15160	**Immobilized Glutathione Agarose,** 10mL

General References

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D'Souza-Schorey, C. and Chavrier, P. (2006). ARF proteins: roles in membrane traffic and beyond. Nat Rev Mol Cell Biol. **7:**347-58.

Yoon, H.Y., *et al.* (2005). In vitro assays of Arf1 interaction with GGA proteins. *Methods Enzymol.* **404:**316-32.

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