Powerful and simple new assay kits for studying signal transduction

Measure activation of small GTPases by isolation via their specific downstream effectors

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Monomeric p21 GTP-binding proteins (small GTPases) serve as molecular switches in regulating a wide range of essential biochemical pathways in eukaryotic cells. The new Thermo Scientific Active Arf1, Arf6 and RalA Pull-Down and Detection Kits measure the activation of small GTPases by preferentially isolating the active GTP-bound form via a specific GST-fusion protein binding domain (PBD). In addition to the GST-PBD fusion protein, each kit includes reagents for generating positive and negative control lysates for GTPase pull-down and detection.

Highlights:

- **Convenient** – no need to express and purify GST-PBD fusion proteins
- **Easy to use** – pull-down conditions are optimized for immediate success, even for first-time users
- **Efficient** – simultaneous incubation of lysate, GST-PBD and glutathione resin in the spin column prevents sample loss
- **Validated** – each kit is functionally tested to ensure quality and performance
- **Sensitive** – optimized antibodies, reagents and Western blotting procedure ensure accurate, quantitative and reproducible results

Small GTPases are integral parts of cell physiology and are involved in several disease states such as cancer and metabolic disorders.[1,2] The Arf superfamily of small GTPases, including Arf1 and Arf6, regulates membrane trafficking pathways. Arf1 regulates assembly of different coat proteins (clathrin) onto budding vesicles on the trans-golgi network and endosomal membranes. Arf6 regulates endocytic membrane traffic, internalization of G-protein coupled receptors, actin remodeling and structural organization at the cell surface.[3] Ral GTPases are close relatives to Ras, sharing 58% sequence identity and having a similar overall structure. RalA regulates various cellular functions such as filopodia formation, endocytosis and exocytosis.[4]

Like other G-proteins, small GTPases cycle between an inactive, GDP-bound state and an active, GTP-bound state. The respective binding domain of the downstream effector for each small GTPase is expressed as a GST-fusion protein which, when immobilized on a resin, is used to pull down the active, GTP-bound GTPase (Figure 1). The pulled-down active GTPase is then detected via Western blot using a specific antibody. Each pull-down kit contains all the necessary components for 30 pull-down assays from 500 µg of cell lysate and a primary antibody for performing a Western blot.

Methods

NIH 3T3 cells were grown in DMEM supplemented with 10% FBS to > 90% confluency in 150 mm culture dishes. Cells were rinsed with TBS and lysed on the plate with 1 ml of Lysis/Binding/Wash Buffer. The clarified cell lysate (500 µg) was treated with 0.1 mM GTPγS (positive control) or 1.0 mM GDP (negative control) in the presence of 10 mM EDTA, pH 8.0 at 30˚C for 15 minutes (to activate or inactivate Arf1 and Arf6) or 45 minutes (to activate or inactivate RalA). The nucleotide exchange reaction was terminated by adding $MgCl_2$ and placing the samples on ice. Treated lysates (500 µg) were incubated at 4˚C for 1 hour with gentle rocking with glutathione resin and GST-GGA3-PBD, to pull down active Arf1 or Arf6, or GST-RalBP1-PBD, to pull down active RalA. To remove the unbound proteins, the resin was briefly centrifuged and washed three times. The samples were eluted and heated at 95˚C for 5 minutes. Half the volume of each elution was analyzed by SDS-PAGE (4-20% polyacrylamide gel) and transferred to PVDF membrane. The active Arf1, Arf6 or RalA was detected by Western blot using a specific GTPase primary antibody. Detection was performed with Goat Anti-Mouse or Anti-Rabbit Antibody (H+L) conjugated with horseradish peroxidase

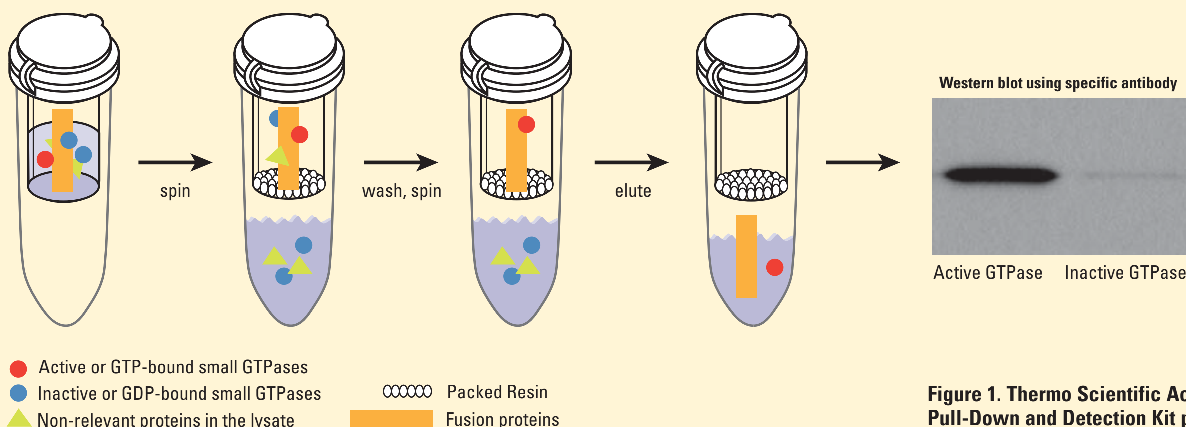

Figure 1. Thermo Scientific Active Arf1, Arf6 and RalA Pull-Down and Detection Kit protocol summary.

(1:10,000 dilution) (Product # 31430 or # 31460), Thermo Scientific SuperSignal West Pico Chemiluminescent Substrate† (Product # 34079) and exposure to X-ray film.

For the pull-down of endogenously active RalA, NIH 3T3 cells were serum-starved in 0.2% FBS for 40 hours. The cells were stimulated with 50 ng/ml platelet-derived growth factor (PDGF) for a 1 hour time course. After treatment, cells were rinsed and lysed as described above. Cell lysate (500 µg) from each time point was incubated with 200 µg of GST-RalBP1-PBD for individual pull-down assays. Active RalA was detected by Western blot as described above.

Results and Discussion

To determine the specificity and function of the Active Arf1, Arf6 and RalA Pull-Down and Detection Kits, NIH 3T3 cell lysate was treated with either GTPγS or GDP to activate or inactivate endogenous GTPases, respectively. GST-GGA3-PBD was used to pull down active Arf1 and Arf6, and GST-RalBP1-PBD was used to pull down active RalA.[5,6] A strong signal from Arf1, Arf6 or RalA was detected by Western blot using GTPγS-treated lysate, while minimal or no signal was detected in the GDP-treated lysate (Figure 2).

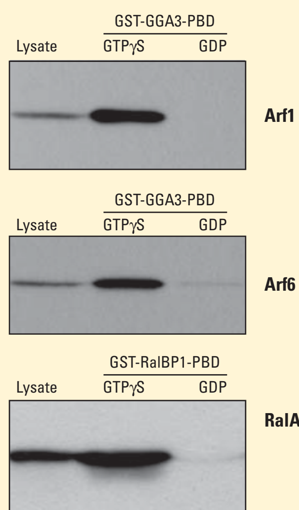

Figure 2. Western blot detection of active Arf1, Arf6 and RalA. NIH 3T3 cell lysate treated with GTPγS or GDP was incubated with GST-GGA3-PBD or GST-RalBP1-PBD and glutathione resin. Half of the eluted samples and 10 µg of lysate were analyzed by Western blot using anti-Arf1, anti-Arf6 or anti-RalA antibodies.

Furthermore, no signal was detected when GST alone was incubated with GTPγS-treated cell lysate (data not shown). The results demonstrate that the Active Arf1, Arf6 or RalA Pull-Down and Detection Kit can specifically monitor each active small GTPase.

The Active Arf1, Arf6 and RalA Pull-Down and Detection Kits are also highly efficient in the pull-down of endogenously active small GTPases after physiological treatments (Figure 3). Here, serum-starved NIH 3T3 cells were stimulated by adding platelet-derived growth factor (PDGF) for various times up to 1 hour. Activation of RalA rapidly increased after the first 5 minutes of PDGF stimulation, and then gradually returned to basal level after 1 hour. These results demonstrate the effectiveness of the Active Arf1, Arf6 and RalA Pull-Down and Detection Kits for monitoring and measuring active small GTPases.

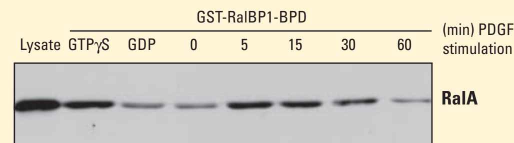

Figure 3. Western blot detection of active RalA stimulated by PDGF. NIH 3T3 cells were serum-starved and then stimulated with PDGF for a time course. Cell lysate (500 µg) was incubated with GST-RalBP1-PBD and glutathione resin. Half of the eluted sample and 10 µg of cell lysate were analyzed by Western blot using anti-RalA antibody.

References:
1. Charest, P. and Firtel, R. (2007). Big roles for small GTPases in the control of directed cell movement. *Biochem J.* **401**, 377-90.
2. Williams, D., *et al.* (2008). Rho GTPases and regulation of hematopoietic stem cell localization. *Methods Enzymol.* **439**, 365-93.
3. D'Souza-Schorey, C. and Chavrier, P. (2006). ARF proteins: Roles in membrane traffic and beyond. *Nat Rev Mol Cell Biol.* **7**, 347-58.
4. Feig, L. (2003). Ral-GTPases: Approaching their 15 minutes of fame. *Trends Cell Biol.* **13**, 419-25.
5. Dell'Angelica, E., *et al.* (2000). GGAs: A family of ADP ribosylation factor-binding proteins related to adaptors and associated with the golgi complex. *J Cell Biol.* **149**, 81-93.
6. Cantor, S., *et al.* (1995). Identification and characterization of Ral-Binding Protein1, a potential downstream target of Ral GTPases. *Mol Cell Biol.* **15**, 4578-84.

Ordering Information:

Product #	Description	Pkg. Size
26185	Active Arf1 Pull-Down and Detection Kit	Kit**
26186	Active Arf6 Pull-Down and Detection Kit	Kit**
26187	Active RalA Pull-Down and Detection Kit	Kit**
89854	Active Rho Pull-Down and Detection Kit	Kit**
89855	Active Ras Pull-Down and Detection Kit	Kit**
89856	Active Rac1 Pull-Down and Detection Kit	Kit**
89857	Active Cdc42 Pull-Down and Detection Kit	Kit**
89872	Active Rap1 Pull-Down and Detection Kit	Kit**
23227	Pierce BCA Protein Assay Reagent Kit	Kit
22660	Pierce 660 nm Protein Assay Reagent	750 ml
78425	Halt* Protease Inhibitor Single-Use Cocktail, EDTA-free (100X)	24 x 100 µl tubes
20291	No-Weigh* Dithiothreitol (DTT)	7.7 mg x 48 tubes
88014	Nitrocellulose Membrane, 0.45 µm	7.9 cm x 10.5 cm
88585	PVDF Membrane, 0.45 µm	7.9 cm x 10.5 cm
20237	GST	1 mg
15160	Immobilized Glutathione Agarose	10 ml
21065	Pierce Background Eliminator Kit	Kit

** Trademark, see Trademark Index on page 35.*
*** Contains sufficient reagents for 30 pull-down reactions*

† Patented, see Patent Index on page 35.