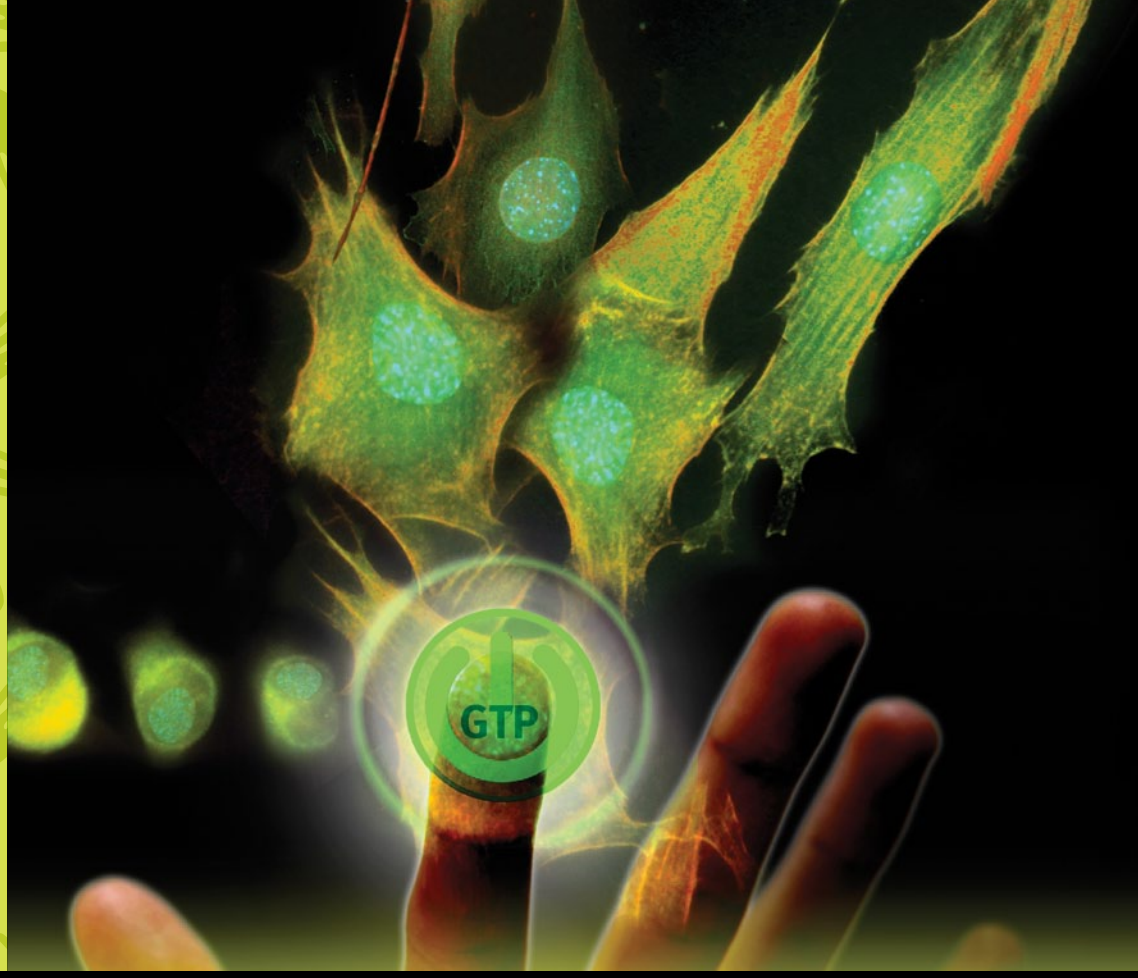

Thermo Scientific GTPase Research Tools

Part of Thermo Fisher Scientific

Thermo SCIENTIFIC

Active GTPase Pull-Down Assays

We offer two different tools to study GTPase biology, one for active GTPase monitoring and one for global GTPase profiling.

The Thermo Scientific Pierce Active GTPase Pull-Down and Detection Kits selectively enrich the active form of a particular GTPase. This method allows researchers to monitor activation levels post treatment.

The Thermo Scientific Pierce GTPase Enrichment Kits label and purify all GTPases present in a sample lysate. This technology can be used for mass spec determination of GTPase content and small molecule inhibitor screening.

GTPases are active when bound to guanosine triphosphate (GTP), and inactive when the triphosphate is hydrolyzed to guanosine diphoshphate (GDP). The Thermo Scientific Active GTPase Pull-Down and Detection Kits enable GTPase activation studies by preferentially enriching their active form.

This pull-down method is based on the affinity of known downstream effector proteins for the active forms of specific GTPases. The respective protein-binding domain (PBD) of these downstream effectors is provided as a GST-fusion protein (Table 1). When immobilized on an agarose resin, the PBD will bind active, GTP-bound GTPase from a cell lysate. The pulled-down active GTPase is detected via Western blotting (Figure 1).

Highlights:

- **Validated** – functionally tested to ensure quality and performance
- **Sensitive** – optimized antibodies, reagents and Western blotting procedure accurately detect changes in GTPase activity levels
- **Convenient** – kits contain controls and all reagents needed to perform and detect 30 pull-downs
- **Easy to use** – conditions are optimized for immediate success in a 2-hour assay
- **Efficient** – spin columns prevent sample loss

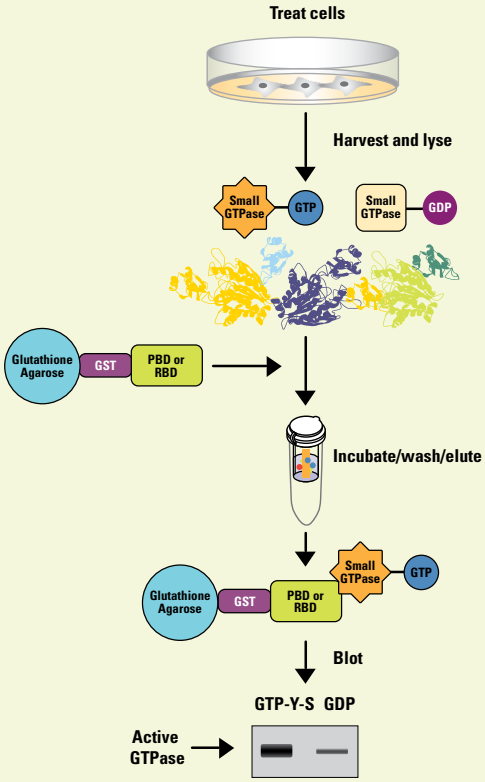

Figure 1. Thermo Scientific Pierce Active GTPase Pull-Down and Detection Kit protocol summary.

Specific

To determine the specificity of the Pierce® Active GTPase Pull-Down and Detection Kits, NIH3T3 cell lysate was incubated with either GTPγS or GDP to activate or inactive endogenous GTPases, respectively (Figure 2). The specific GST-PBD or RBD was used to pull down active Rho, Ras, Rac1, Cdc42, Rap1, Arf1 or Arf6. A strong signal is detected in the GTPγS-treated lysate; however, minimal or no signal is detected in the GDP-treated lysate. These results illustrate the specificity of the PBD for active GTPases.

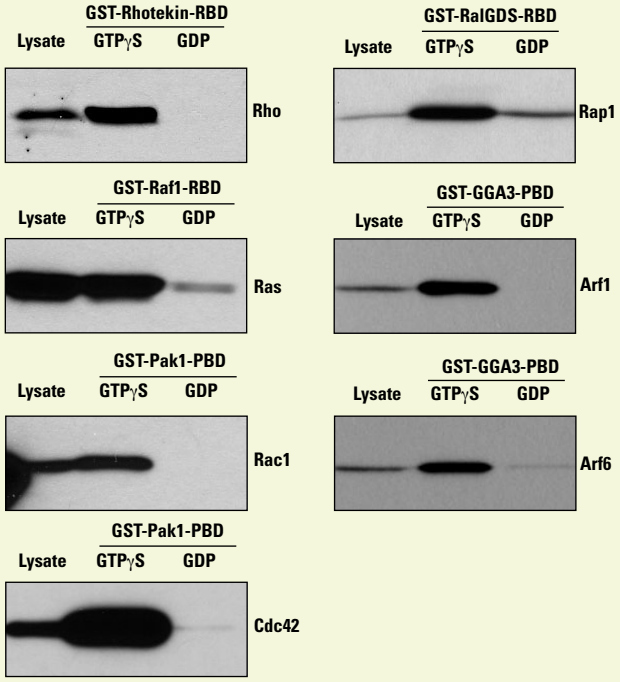

Figure 2. Specific detection of active Rho, Ras, Rac1, Cdc42, Rap1, Arf1 and Arf6 by Western blotting. NIH3T3 cell lysate treated with GTPγS or GDP was incubated with the appropriate GST-PBD and immobilized glutathione resin. Eluted samples and a portion of the lysate were analyzed by Western blot using GTPase-specific antibodies provided in the kit.

Table 1. Each active GTPase kit includes a GST fusion of the protein-binding domain.

GTPase	Downstream effector binding domain	Cellular function
Rho	GST-Rhotekin-RBD	Filopodia, lamellipodia formation, and stress fibers[1]
Ras	GST-Raf1-RBD	Cell proliferenation/differentiation[2]
Rac1	GST-Pak1-PBD	Filopodia, lamellipodia formation, and stress fibers[1]
Cdc42	GST-Pak1-PBD	
Rap1	GST-RalGDS-RBD	Cell proliferenation/differentiation[3]
Arf1	GST-GGA3-PBD	Assembly of coat proteins onto budding vesicles on trans-golgi network and endosomes[4,5]
Arf6	GST-GGA3-PBD	Membrane traffic, actin remodeling and structural organization at the cell surface[4,5]

We compared Active Ras enrichment and detection using an active GTPase pull-down kit available from Millipore, an ELISA based method from Active Motif and the Thermo Scientific Pierce Active Ras Pull-down and Detection kit (Figure 3). The Thermo Scientific kit showed better Active GTPase enrichment and detection. The ELISA method gave high background noise which made measuring activity levels difficult.

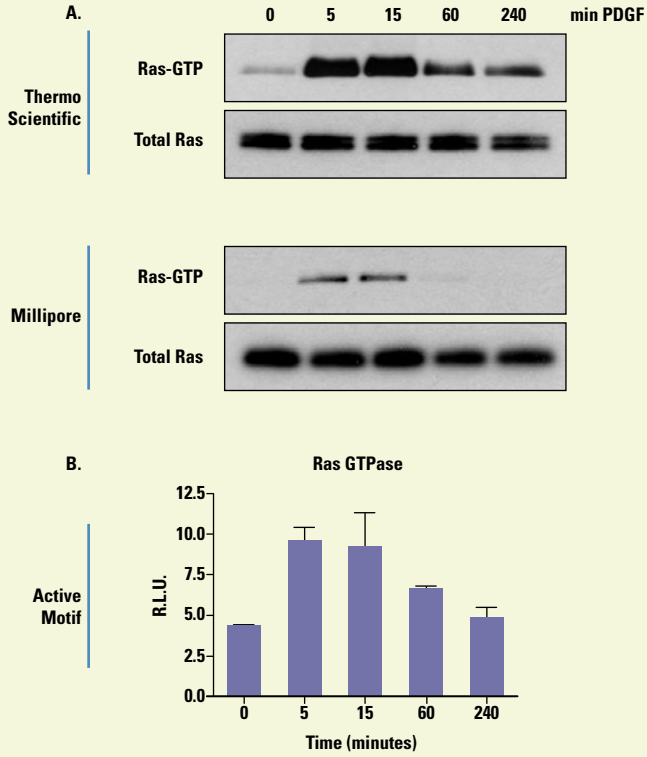

Figure 3. Thermo Scientific Pierce Active Ras Pull-down and Detection kit outperforms competitors. NIH3T3 cells were serum-starved and stimulated with 50ng/mL platelet derived growth factor (PDGF) over a time course. Cells were harvested at each time point. **Panel A:** Cell lysates (500µg) were used in active Ras pull-down assays from Thermo Scientific and Millipore performed according to the manufacturers instructions. The top blots in each set represent Ras-GTP. The bottom blots are shown as a loading control (10µg of total lysate). 10 second exposures are shown. **Panel B:** 25µg of cell lysate (n=3) was analyzed using the Pierce Active Motif Ras GTPase Chemi ELISA.

References

1. Van Aelst, L. and D'Souza-Schorey, C. (1997). Rho GTPases and signaling networks. *Genes Dev* **11**:2295-322.
2. Ehrhardt, A., *et al.* (2002). Ras and relatives - job sharing and networking keep an old family together. *Exp Hematol* **30**:1089-106.
3. Posern, G., *et al.* (1998). Activity of Rap1 is regulated by bombesin, cell adhesion and cell density in NIH3T3 fibroblasts. *J Bio Chem* **273**:24297-300.
4. Yoon H.Y., *et al.* (2005). *In vitro* assays of Arf1 interaction with GGA proteins. *Methods Enzymol* **404**:316-32.
5. D'Souza-Schorey, C. and Chavrier, P. (2006). ARF proteins: roles in membrane traffic and beyond. *Nat Rev Mol Cell Biol* **7**:347-58.

Compatibility

To test the compatibility of the Pierce Active GTPase Pull-down and Detection kits with different species, the pull-down of endogenous active small GTPases after growth factor or serum stimulations was performed in a variety of cell types (Figure 4). Changes in the GTPase activities was detected in time-course studies. Because total GTPase levels in each lysate are constant, the amount of GTPase pulled down in each assay reflects activation rather than changes in GTPase expression levels.

The activity profiles detected are similar to those reported in the literature. These results demonstrate the effectiveness of the Pierce Active GTPase Pull-Down and Detection Kits for monitoring sensitive changes in activity. These kits can be used with different species, including human, mouse, rat and canine cell types.

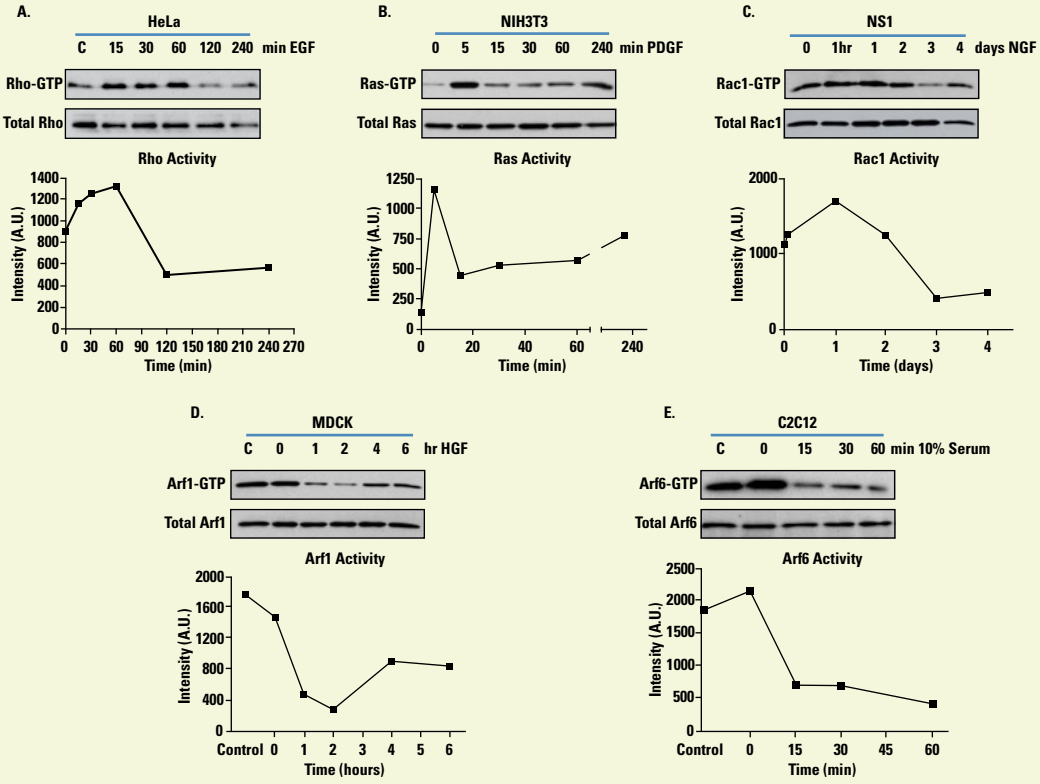

Figure 4. Specific, induced changes in the level of active GTPases from a variety of cell types are easily monitored by the pull-down assay. In each panel, the top Western blot shows the level of active GTPase isolated by the pulldown assay; the lower Western blot shows the total amount of expressed GTPase in the lysate. Densitometry was performed on the Western blots and plotted graphically for each system. **Panel A:** HeLa (human) cells stimulated with EGF. **Panel B:** NIH3T3 (murine) cells stimulated with PDGF. **Panel C:** NS1 (rodent) cells stimulated with NGF. **Panel D:** MDCK (canine) cells stimulated with HGF. **Panel E:** C2C12 (murine) cells stimulated with serum.

Application: Neuronal Profiling

The Pierce Active GTPase Pull-Down and Detection Kits can be used to monitor activity of multiple GTPases in the same experiment. We stimulated neuronal NS-1 cells with Neuronal Growth Factor (NGF) and studied Rho and Ras family GTPase activity (Figure 5). Active GTPase activity was assessed by a functional pull-down assay using a GST fusion of the downstream effector protein that only binds the active form of the GTPase. The spatial distribution of active GTPases was determined by immunofluorescent staining using the GST-PBD protein and anti-GTPase antibody supplied in the kit (Figure 6). Activity levels peaked at two days post treatment, and immunofluorescent staining showed localized activity levels in neurite outgrowths.

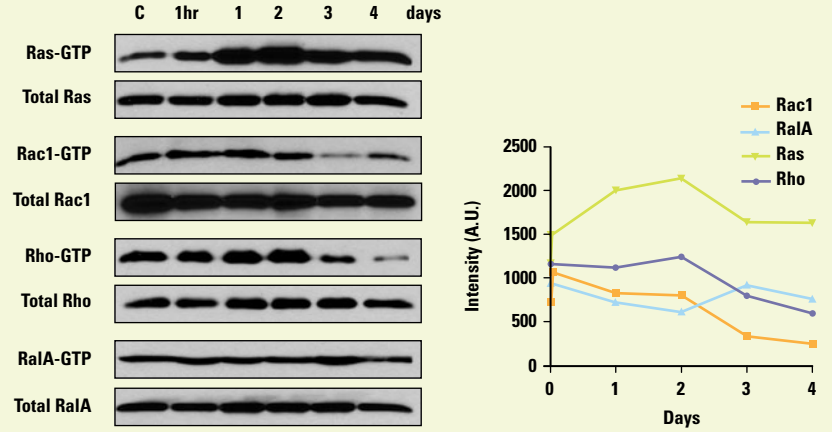

Figure 5. Assay of GTPase activity by functional pull-down. Active GTPases were purified from NGF-stimulated NS-1 cells at different time post treatment. Samples were immunoblotted and spot densitometry was performed on each scanned blot and normalized to scale. The graph summarizes the induction of Ras, Rac1, Rho, and RalA for a 4-day period.

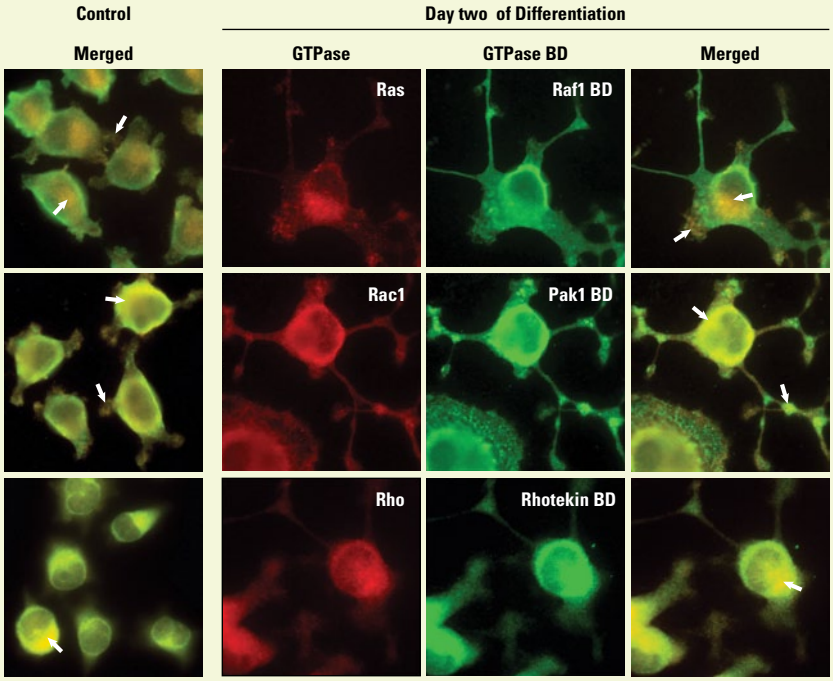

Figure 6. Use of GTPase binding domains as antibody alternatives localizes GTPases activity in differentiated neuronal cells. NS-1 cells were grown and treated with NGF. Images of GTPase and GTPase-binding domain (BD) staining of day 2 differentiated cells are monochrome. Merged images of non-treated (control) and day 2 differentiated cells are in color. GTPases were detected using DyLight 549-conjugated secondary antibodies. TheGTPase effector binding proteins were detected using DyLight 488-conjugated anti-GST antibody. Arrows denote areas of activity as seen by colocalization of the GTPase and the GTPase BD.

Ordering Information

Product #	Description	Pkg. Size
16116	Active Rho Pull-Down and Detection Kit	30-rxn kit†
16117	Active Ras Pull-Down and Detection Kit	30-rxn kit†
16118	Active Rac1 Pull-Down and Detection Kit	30-rxn kit†
16119	Active Cdc42 Pull-Down and Detection Kit	30-rxn kit†
16120	Active Rap1 Pull-Down and Detection Kit	30-rxn kit†
16121	Active Arf1 Pull-Down and Detection Kit	30-rxn kit†
16122	Active Arf6 Pull-Down and Detection Kit	30-rxn kit†

Kit Contents		
	GST Fusion Protein of Specific Binding Domain	1 vial
	Glutathione Agarose Resin	3mL
	GTPγS (100X)	50µL
	GDP (100X)	50µL
	Lysis/Binding/Wash Buffer	100mL
	GTPase-Specific Primary Antibody	1 vial
	SDS-PAGE Sample Loading Buffer (2X)	1.5mL
	Spin Cups	30 cups
	Collection Tubes	90 tubes

† Kits will be shipped as a dry ice package and a wet ice package. Please review product guidelines for proper storage.

Global GTPase Profiling

Thermo Scientific Pierce GTPase Enrichment Kits utilize GTP Probes to covalently bind to the GTP binding sites of all GTPases and G-protein coupled receptor GTPase subunits. These probes feature a desthiobiotin (biotin analog) that can be used to selectively enrich, identify and profile target enzyme classes across samples or assess the specificity and affinity of enzyme inhibitors (Figure 1).

Highlights:

- Broad enrichment of GTP binding proteins from tissues, cells and subcellular proteomes
- Enrichment of enzymes based on function
- Profile dozens of inhibitor targets

Broad Enrichment

For global profiling of GTPases in a biological sample, the Pierce GTPase Enrichment Kit with GTP probe can be used. These kits label GTP-binding pockets with nucleotide analogues that possess a desthiobiotin moiety (Figure 2). Active-site labeling is assessed by either Western blot or mass spectrometry (MS). For the Western blot workflow, desthiobiotin-labeled proteins are enriched for SDS-PAGE analysis and subsequent detection with specific antibodies (Figure 3). For the MS workflow, desthiobiotin-labeled proteins are reduced, alkylated and enzymatically digested to peptides. Only the desthiobiotin-labeled, active-site peptides are enriched for analysis by LC-MS/MS (Table 1). Both workflows can be used for determining inhibitor target binding, but only the MS workflow can identify global inhibitor targets and off targets.

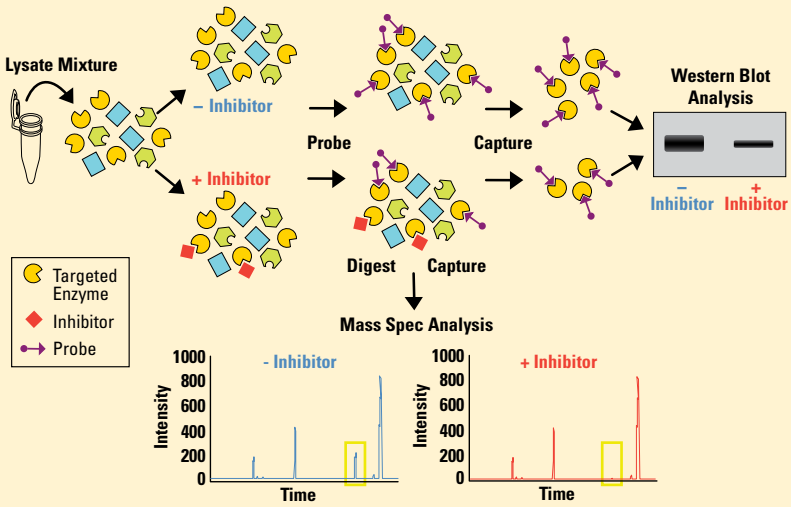

Figure 1. Assessment of active-site labeling is accomplished by Western blot or mass spectrometry. For the Western blot workflow, desthiobiotin-labeled proteins are enriched, analyzed by SDS-PAGE and detected with specific antibodies. For the MS workflow, desthiobiotin-labeled proteins are reduced, alkylated and enzymatically digested. Only the desthiobiotin-labeled, active-site peptides are enriched for LC-MS/MS analysis. Both workflows can be used to determine inhibitor target binding, but the MS workflow also can identify global inhibitor targets and off-targets and provide higher throughput for quantitative assays.

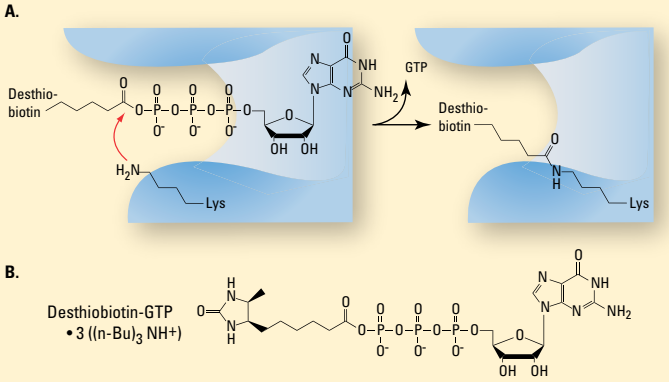

Figure 2. Mechanism and chemical structures of Thermo Scientific Pierce Active Site Probes for GTPases. Panel A: Nucleotide analogues bind to the active sites of GTPases and the biotin affinity tag is irreversibly transferred to highly conserved lysine residues in the active site. **Panel B:** Desthiobiotin is attached to the GTP nucleotide through a labile acyl phosphate linkage, allowing efficient desthiobiotin label transfer to amines near the active site of GTPases. Desthiobiotin binding to streptavidin is easily reversible under acidic elution conditions, allowing high recovery of labeled proteins and peptides.

Selective

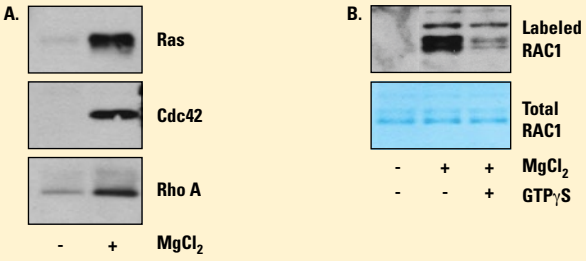

Figure 3. Desthiobiotin-GTP probe specifically labels small GTPases. Panel A: A549 cell lysates (500µg) were treated with (+) or without (-) 20mM of $MgCl_2$ after labeling with 20µM of desthiobiotin-GTP probe. Desthiobiotin-labeled proteins were denatured and enriched using streptavidin agarose before separation by SDS-PAGE and Western blotting with specific GTPase antibodies. **Panel B**: Recombinant Rac1 was treated with GTPγS before labeling with desthiobiotin-GTP probe. Labeling was performed in the presence (+) or absence (-) of 20mM $MgCl_2$. Samples were separated by SDS-PAGE and analyzed by Western blot (Labeled) to detect biotinylation of the active site. Thermo Scientific GelCode Blue Stain Reagent (Total) was used to stain a duplicate gel to show equal loading.

Table 1. List of GTPases from human cell lysates identified by mass spectrometry after labeling and enrichment using desthiobiotin-GTP probe.

	Total of GTPases per family
Rab family	38
Ras family	9
Arf family	8
Rho family	5
Gα family	4
Sar1 family	2

Data provided by ActivX Biosciences Inc.

Ordering Information

Product #	Description	Pkg. Size
88314	Pierce GTPase Enrichment Kit with GTP Probe *Sufficient reagents for 16 pull-down reactions.*	Kit
88315	ActivX® Desthiobiotin-GTP Probe	16 x 12.9µg

Learn more with Thermo Scientific Pierce Technical Handbooks

Protein Interaction Technical Handbook (1601945)

Protein Purification Technical Handbook (1602015)

www.thermoscientific.com/pierce

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