GTPases are active when bound to guanosine triphosphate (GTP), and inactive when the triphosphate is hydrolyzed to guanosine diphosphosphate (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).

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 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 GDP 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.

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.

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

<table>
<thead>
<tr>
<th>GTPase</th>
<th>Downstream effector binding domain</th>
<th>Cellular function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rho</td>
<td>GST-Rhotekin-RBD</td>
<td>Filopodia, lamellipodia formation, and stress fibers</td>
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<tr>
<td>Ras</td>
<td>GST-Raf1-RBD</td>
<td>Cell proliferation/differentiation</td>
</tr>
<tr>
<td>Rac1</td>
<td>GST-Pak1-PBD</td>
<td>Filopodia, lamellipodia formation, and stress fibers</td>
</tr>
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<td>Cdc42</td>
<td>GST-Pak1-PBD</td>
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<tr>
<td>Rap1</td>
<td>GST-RalGDS-RBD</td>
<td>Cell proliferation/differentiation</td>
</tr>
<tr>
<td>Arf1</td>
<td>GST-GGA3-PBD</td>
<td>Assembly of coat proteins onto budding vesicles on trans-Golgi network and endosomes</td>
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<tr>
<td>Arf6</td>
<td>GST-GGA3-PBD</td>
<td>Membrane traffic, actin remodeling and structural organization at the cell surface</td>
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</table>

References
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.
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 assayed 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.

**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 assayed 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.

**Ordering Information**

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<td>16122</td>
<td>Active Arf6 Pull-Down and Detection Kit</td>
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**Kit Contents**

- GST Fusion Protein of Specific Binding Domain: 1 vial
- Glutathione Agarose Resin: 3mL
- GTPyS (100X): 50µL
- GDP (100X): 50µL
- Lysis/Wash Buffer: 100mL
- GSTase-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.

Selectivity

A. Ras
Cdc42
Rho A
B. Labeled RAC1
Total RAC1
- + + MgCl₂
- - + GTP-γS

Figure 3. Desthiobiotin-GTP probe specifically labels small GTPases. Panel A: A549 cell lysates (500µg) were treated with (+) or without (-) 20mM of MgCl₂ 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 GTP-γS before labeling with desthiobiotin-GTP probe. Labeling was performed in the presence (+) or absence (-) of 20mM MgCl₂. 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.

<table>
<thead>
<tr>
<th>Total of GTPases per family</th>
<th>Rab family</th>
<th>Ras family</th>
<th>Arf family</th>
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<th>Gα family</th>
<th>Sar1 family</th>
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<td>9</td>
<td>8</td>
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Data provided by ActivX Biosciences Inc.

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<td>Sufficient reagents for 16 pull-down reactions.</td>
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