INSTRUCTIONS

Active Rap1 Pull-Down and Detection Kit

16120 2273.2

Number	Description
16120	**Active Rap1 Pull-Down and Detection Kit**, contains sufficient reagents for 30 pull-down reactions

Kit Contents:

Box 16120X (these items ship together on dry ice; upon receipt store at -70°C):

GST-RalGDS-RBD Fusion Protein, 1 × 600µg, contains 1-2mg/mL in 50mM Tris•HCl, pH 7.2, 150mM NaCl, 0.5% Triton® X-100, 5mM $MgCl_2$, 1mM DTT, protease inhibitors and 10% glycerol; ~40kDa; GST-RalGDS-RBD interacts with Rap1 from human, mouse, and rat origin, store at -70°C

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

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

Box 16120Y (these items ship together with an ice pack; upon receipt store products as directed):

Glutathione Resin, 3.0mL, supplied as a 50% slurry containing 0.05% sodium azide, store at 4°C

1X Lysis/Binding/Wash Buffer, 100mL, 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, 125mM Tris•HCl, pH 6.8, 2% glycerol, 4% SDS (w/v) and 0.05% bromophenol blue, store at 4°C

Anti-Rap1 Antibody, 50µL (5 units), rabbit monoclonal IgG; Anti-Rap1 antibody reacts with Rap1A and Rap1B of human, rat, and mouse, store at -20°C.

> **Note:** One unit of Anti-Rap1 antibody is defined as the amount of antibody required to efficiently detect Rap1 in 40µg NIH3T3 whole cell lysate by Western blotting (8.5 × 7.5cm membrane).

Spin Cups, 30 each, maximum capacity 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 Rap1 Pull-Down and Detection Kit is a simple and fast tool to monitor Rap1 small GTPase activation. The kit provides a GST-fusion protein of the Rap1-binding domain (RBD) from human RalGDS along with glutathione agarose resin to specifically pull down active Rap1 and an anti-Rap1 antibody for Western blot detection. Also included are two control nucleotides, GTPγS and GDP, which can be used to generate positive and negative control lysates, respectively. Each kit is functionally tested to ensure component performance.

Small GTP-binding proteins (or GTPases) serve as molecular switches in signaling transduction pathways. The small GTPase Rap1 (~24kDa) is a member of the Ras superfamily of small GTPases. Rap1 was originally characterized as an antagonist of Ras-induced cell transformation and has been implicated in regulation of cell morphogenesis and cell-cell adhesion. Rap1 is activated by a diverse array of extracellular stimuli and secondary messengers. The resulting signal cascade can result in both the activation and inhibition of ERK (extracellular signal-regulated kinase). Like other small GTPases, Rap1 is active when bound to GTP and inactive when bound to GDP.

Important Product Information

- Rap1-GTP is quickly hydrolyzed to Rap1-GDP; prepare lysate just before use for each assay, if possible. Otherwise, immediately freeze the lysate at -70°C after preparation.
- For optimal pilot experiments, use 500µg to 1 mg of total lysate per assay.
- Lysis/Binding/Wash buffer is compatible with Thermo Scientific Pierce BCA (Product No. 23227) and Pierce 660nm (Product No. 22660) Protein Assays but not the Coomassie (Bradford) Protein Assay.
- For best results always use protease inhibitors for lysis, and keep lysates on ice between steps.
- 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 will require optimization.

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)
- BSA, Fraction V
- Nonfat Dry Milk
- Pierce Goat Anti-Rabbit IgG (H+L), Peroxidase Conjugated (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)
- Electrophoresis Apparatus
- Variable-speed Bench-top Microcentrifuge

Procedure for Pull-Down and Detection of GTP-Rap1

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 Lysis/Binding/Wash Buffer per 75cm^2 flask or 0.3-0.5mL Lysis/Binding/Wash Buffer 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 and then 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)

Perform the following treatments, GTPγS (positive control) and GDP (negative control) to ensure the pull-down procedures are working properly. Use 500µg of cell lysate for each treatment. For best results, aliquot GTPγS and GDP at first use to minimize freeze/thaw cycles.

1. For 500µL lysate, add 10µL 0.5M EDTA pH 8.0 (for a final concentration of 10mM) and vortex the sample.
2. Add 5µL of 10mM GTPγS (for a final concentration of 0.1mM) or 5µL 100mM GDP (for a final concentration of 1mM) and vortex the sample.
3. Incubate the mixture at 30°C for 30 minutes with constant agitation.
4. Terminate the reaction by placing the sample on ice and adding 32µL of 1M MgCl2 (for a final concentration of 60mM) and vortex the sample.

C. Affinity Precipitation of Activated Rap1

1. Save a sample of the cell lysate for protein assay using the Pierce BCA or 660nm Protein Assay.
2. Place a spin cup into a collection tube for each sample.
3. Swirl the bottle of Glutathione Resin to thoroughly resuspend the agarose beads. Add 100µL of the 50% resin slurry to the spin cup with collection tube. Centrifuge the tubes at 6000 × g for 10-30 seconds.
4. Discard the flow-through. Add 400µL of Lysis/Binding/Wash Buffer to each tube with resin. Invert the tubes gently several times. Centrifuge the tubes at 6000 × g for 10-30 seconds. Discard the flow-through.
5. Thaw the GST-RalGDS-RBD on ice and immediately make 20µg aliquots. Store aliquots for later use at -70°C.
6. Add 20µg of GST-RalGDS-RBD to the spin cup containing the glutathione resin.
7. Immediately transfer up to 700µL of the cell lysate (containing at least 500µg of total proteins) to the spin cup, close the cap and vortex the sample.

8. Seal cap of the collection tube with laboratory film to prevent leakage, which may result from the presence of detergent in the lysate, and vortex the sample.
9. Incubate the reaction mixture at 4°C for 1 hour with gentle rocking.
10. Centrifuge the spin cup with collection tube at 6000 × g for 10-30 seconds.
11. Remove the laboratory film and transfer the spin cup to a new collection tube.
12. To wash resin, add 400µL of Lysis/Binding/Wash Buffer, invert the tube three times, and centrifuge at 6000 × g for 10-30 seconds. Decant the buffer. Repeat this wash step two additional times.
13. Transfer the spin cup to a new collection tube.
14. 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., mix 2.5µL of β-mercaptoethanol to 50µL of 2X SDS Sample Buffer), or by adding dithiothreitol (DTT) to a final concentration of 200mM.
15. Add 50µL 2X reducing sample buffer to the resin. Vortex the sample and incubate at room temperature for 2 minutes.
16. Centrifuge the tube at 6000 × g for 2 minutes. Remove and discard the spin cup containing the resin.
17. Heat the eluted samples for 5 minutes at 95-100°C. Samples may be electrophoresed on a gel or stored at -20°C until use. Apply at least 25µL per lane for a 10 × 10cm mini-gel (12% or 4-20% acrylamide gel provides the best separation).

D. Western Blot Analysis

Notes:

- This procedure has been optimized for use with SuperSignal West Pico Chemiluminescent Substrate (see Important Product Information section).
- Include unfractionated cell lysate as a control to verify that the Western blot analysis is functioning properly.
- Perform all blocking, probing and washing incubation steps using constant agitation.

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

   Note: Using a PVDF membrane may result in a high background signal.
2. Block the membrane in TBS containing 3% BSA at room temperature for 2 hours.

   Note: Do not block with nonfat dry milk. Nonfat dry milk will significantly reduce the Rap1 signal on the Western blot.
3. Rinse the membrane with TBS containing 0.05% Tween-20 (TBST).
4. Prepare a solution containing the Anti-Rap1 Antibody (1:1000 dilution) in 5% BSA and 0.1% NaN_3 in TBST. An example of a 1:1000 dilution is to add 10µL of the stock antibody solution to 10mL of buffer.
5. Incubate the membrane in the anti-Rap1 antibody solution at 4°C overnight.

   Note: If the number of pull-down reactions per blot is low, the diluted anti-Rap1 antibody solution can be re-used up to three times with no performance loss. Store the diluted anti-Rap1 antibody solution at 4°C for up to two months.
6. Wash the membrane five times for 5 minutes each with TBST.
7. Dilute the anti-rabbit IgG-HRP conjugate in TBST containing 5% BSA [e.g., if using Pierce Goat Anti-Rabbit IgG (H+L), Peroxidase Conjugated (Product No. 31460), dilute at 1:20,000 to 1:100,000].
8. Incubate the membrane in the diluted anti-rabbit IgG HRP conjugate at room temperature for 1 hour.
9. Wash the membrane five times for 5 minutes each with TBST.
10. Incubate the membrane with chemiluminescent substrate at room temperature (e.g., SuperSignal West Pico Chemiluminescent Substrate).
11. Immediately expose the membrane to X-ray film or a CCD camera.

    Note: The Rap1 band is located at ~24kDa.

Troubleshooting

Problem	Cause	Solution
No activated Rap1 isolated	No activated Rap1 was present in lysates	Include GTPγS-treated lysate as positive control for pull-down
	Insufficient activated Rap1	Increase the amount of lysate used for detection
	GST-RalGDS-RBD was not added	Add GST-RalGDS-RBD to the reactions
	Degraded GST-RalGDS-RBD	Avoid multiple freeze/thaw cycles of GST-RalGDS-RBD
	Degraded lysate proteins	Add protease inhibitors to the Lysis/Binding/Wash Buffer before lysing the cells
	Incorrect secondary antibody was used for detection	Use goat anti-rabbit IgG
	Detection system was 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 were no longer functional	Aliquot GTPγS or GDP after first thaw and store at -70°C; avoid repeated freeze/thaw cycles of the resuspended solution
	Incorrect concentration of EDTA or $MgCl_2$	Prepare new solutions with correct concentrations
Western blot resulted in high background	Inadequate blocking and/or washing	Optimize blocking; increase length, number or volume of washes
	Secondary antibody concentration was too high	Use the secondary antibody at a higher dilution

Additional Information

Rap1 is active when bound to GTP and inactive when bound to GDP. Upon binding to GTP, Rap1 interacts with downstream effectors such as RalGDS. Furthermore, binding of Rap1 to the Rap1-binding domain (RBD) from RalGDS inhibits intrinsic and GAP-enhanced GTPase activity of Rap1. Therefore, the RalGDS-RBD can be used as a probe to specifically isolate active or GTP-Rap1 (Figure 1).

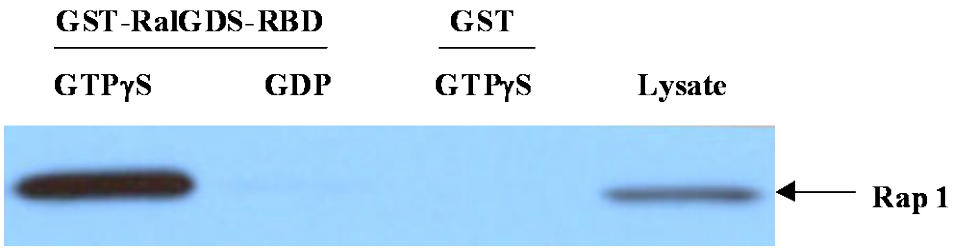

Figure 1. Western blot of control reactions. NIH3T3 cell lysates (500µg) were treated *in vitro* with GTPγS or GDP to activate or inactivate Rap1 (refer to optional section B). The lysates (500µg) were then incubated with GST-RalGDS-RBD and a Glutathione Resin. GTPγS-treated lysate was also incubated with GST alone in the presence of a Glutathione Resin (negative control). Fifty percent of the volume of the eluted samples (24µL) and 30µg of cell lysate were separated by 4-20% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with Anti-Rap1 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 performed with SuperSignal West Pico Chemiluminescent Substrate (Product No. 34080) and followed by exposure to X-ray film.

Related Thermo Scientific Products

25200-44	**Precise™ Protein Gels** (see catalog or website for a complete listing)
21065	**Pierce Background Eliminator Kit**
23236	**Pierce Coomassie Plus (Bradford) Protein Assay Reagent**
23227	**Pierce BCA Protein Assay Reagent Kit**
22660	**Pierce 660nm Protein Assay Reagent,** 750mL
28320	**Surfact-Amps™ 20 Detergent Solution**, 6 × 10mL
28379	**BupH™ Tris Buffered Saline Packs,** 10 packs, each makes 500mL
28358	**Tris Buffered Saline, 20X**, 500mL
78425	**Halt™ Protease Inhibitor Single-Use Cocktail, EDTA-free (100X),** 24 × 100µL microtubes
34079	**SuperSignal West Pico Chemiluminescent Substrate**, 500mL
34090	**CL-XPosure™ Film (5" × 7" sheets)**, 100 sheets/pkg
34091	**CL-XPosure Film (8" × 10"),** 100 sheets/pkg
20291	**Dithiothreitol, (DTT) No-Weigh™ Format,** 7.7 mg DTT/Tube × 48 tubes
88014	**Nitrocellulose Membrane**, 0.45µm, 7.9cm × 10.5cm
88585	**PVDF Membrane,** 0.45µm, 7.9cm × 10.5cm
21059	**Restore® Western Blot Stripping Buffer,** 500mL

General References

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Knox, A.L. and Brown, N.H. (2002). Rap1 GTPase regulation of adherens junction positioning and cell adhesion. *Science* **295:1**285-8.

Stork, P.J.S. and Schmitt, J.M. (2002). Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. *Trends in Cell Biology* **12(6):**258-66.

Zwartkruis, F.J. and Bos, J.L. (1999). Ras and Rap1: two highly related small GTPases with distinct function. *Exp Cell Res* **253:**157-65.