INSTRUCTIONS

Active Rho Pull-Down and Detection Kit

16116 2267.0

Number	Description
16116	**Active Rho Pull-Down and Detection Kit**, contains sufficient reagents for 30 pull-down reactions

Kit Contents:

Box 16116X (these items ship together on dry ice; upon receipt store at -70°C):

GST-Rhotekin-RBD, 3 × 4mg, contains 5-6mg/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; ~36kDa; GST-Rhotekin-RBD interacts with Rho from human, mouse and possibly 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 16116Y (these items ship together with an ice pack; upon receipt store items as directed):

Anti-Rho Antibody, 75µL (5 units**)**, rabbit IgG; Anti-Rho antibody reacts with RhoA, RhoB and RhoC of human, rat and mouse; store at -20°C. Note: One unit of Anti-Rho antibody is defined as the amount of antibody required to efficiently detect Rho in 20µg NIH3T3 whole cell lysate by Western blotting (8.5 × 7.5cm membrane).

Stabilized Goat Anti-Rabbit IgG (H+L), Peroxidase Conjugated, 2mL, 10 µg/mL, store at 4°C

Glutathione Resin, 3.0mL, supplied as 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

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

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

Table of Contents

Introduction 2
Important Product Information 2
Additional Materials Required 2
Procedure for Active Rho Pull-Down and Detection 3
A. Cell Lysis 3
B. *In vitro* GTPγS or GDP Treatment (Optional) 3
C. Affinity Precipitation of Activated Rho 3
D. Western Blot Analysis 4
Troubleshooting 5
Additional Information 5
Related Products 6
General References 6

Introduction

The Thermo Scientific Active Rho Pull-Down and Detection Kit is a simple and fast tool to monitor Rho small GTPase activation. The kit provides a GST-fusion protein of the Rhotekin-binding domain (RBD) along with glutathione agarose resin to specifically pull down active Rho and an anti-Rho 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. Rho (~24kDa), a small GTPase, regulates stress fiber formation, focal adhesion and cell migration. Furthermore, Rho has also been shown to stimulate gene transcription and to participate in Ras-induced cell transformation indicating that it plays an important role in cell growth. Like other small GTPases, Rho is active when bound to GTP and inactive when bound to GDP.

Important Product Information

- **Note: The primary antibody has been changed.** Earlier versions of this kit used a mouse anti-Rho primary antibody. The kit now uses a rabbit anti-Rho antibody and also includes Stabilized Goat Anti-Rabbit IgG (H+L), HRP conjugated as the secondary antibody. When performing the Western blot step, make sure to use the appropriate secondary antibody for the species of primary antibody used.
- Rho-GTP is quickly hydrolyzed to Rho-GDP; use fresh lysate for each assay, if possible. Otherwise, immediately freeze the lysate at -70°C after preparation.
- 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 Bradford Protein Assay.
- For best results always use protease inhibitors when lysing cells, and keep lysates on ice between steps.
- For optimal pilot experiments, use 500µg to 1 mg of total lysate per assay.
- For measuring the activation of endogenous Rho, use at least 1mg of total lysate per assay.
- For best results when performing the Western blotting procedure, use the stabilized Goat Anti-Rabbit IgG (H+L), HRP conjugated that is provided and Thermo Scientific SuperSignal West Pico Chemiluminescent Substrate (Product No. 34080). 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 Cocktail, EDTA-Free, Product No. 87785)
- 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 (Product No. 28320)
- BSA, Fraction V
- Nonfat Dry Milk
- 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 $MgCl_2$
- Electrophoresis Apparatus
- Variable-speed Bench-top Microcentrifuge

Procedure for Active Rho 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 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 15 minutes with constant agitation.
4. Terminate the reaction by placing the sample on ice and adding 32µL of 1M $MgCl_2$ (for a final concentration of 60mM) and vortex the sample.

C. Affinity Precipitation of Activated Rho

1. Save a sample of the cell lysate for protein assay using the Pierce BCA Protein Assay 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 6,000 × *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 6,000 × *g* for 10-30 seconds. Discard the flow-through.
5. Thaw the GST-Rhotekin-RBD on ice and immediately make 400µg aliquots. Store aliquots for later use at -70°C.
6. Add 400µg of GST-Rhotekin-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 6,000 × *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 6,000 × *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 6,000 × *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.
18. Apply at least 25µL per lane for a 10 × 10cm mini-gel (12% acrylamide gel provides the best separation).

D. Western Blot Analysis

Notes:

- This procedure has been optimized for use with SuperSignal West Pico Chemiluminescent Substrate.
- 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.
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 Rho signal on the Western blot
3. Rinse the membrane with TBS containing 0.05% Tween-20 (TBST) for 5 minutes.
4. Prepare a solution containing 15µL of the Anti-Rho Antibody in 10mL of 3% BSA in TBST.
5. Incubate the membrane in the anti-Rho antibody solution at 4°C overnight.
6. Wash the membrane five times for 5 minutes each with TBST.
7. Dilute the Stabilized Goat Anti-Rabbit IgG (H+L), HRP Conjugated by adding 20µL to 10mL (1:500 dilution) of 5% nonfat dry milk in TBST.

   Note: Earlier versions of this kit used a mouse anti-Rho primary antibody. The kit now uses a rabbit anti-Rho antibody and also includes Stabilized Goat Anti-Rabbit IgG (H+L), HRP Conjugated as the secondary antibody. Please verify that you apply the appropriate secondary antibody for the species of primary antibody used.

   Note: Ensure the dry milk is completely dissolved in TBST (e.g., mix the milk in TBST on a stir-plate for 30 minutes at room temperature), otherwise the milk residuals can cause background for the Western blot.
8. Incubate the membrane in the diluted Goat 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 Rho band is located at ~24kDa.

Troubleshooting

Problem	Cause	Solution
No activated Rho isolated	No activated Rho present in lysates	Include GTPγS-treated lysate as positive control for pull-down
	Insufficient activated Rho	Increase the amount of lysate used for detection
	GST-Rhotekin-RBD was not added	Add GST-Rhotekin-RBD to the reactions
	Degraded GST-Rhotekin-RBD	Avoid multiple freeze/thaw cycles of GST-Rhotekin-RBD
	Degraded lysate proteins	Add protease inhibitors to the Lysis/Binding/ Wash Buffer before lysing the cells
	Incorrect secondary antibody used for detection	Use goat anti-rabbit IgG (make sure to use the appropriate secondary antibody for the species of primary antibody used)
	Detection system is not functioning properly or requires optimizations	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 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	Consult the instructions for the detection system being used
	Secondary antibody concentration is too high	

Additional Information

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

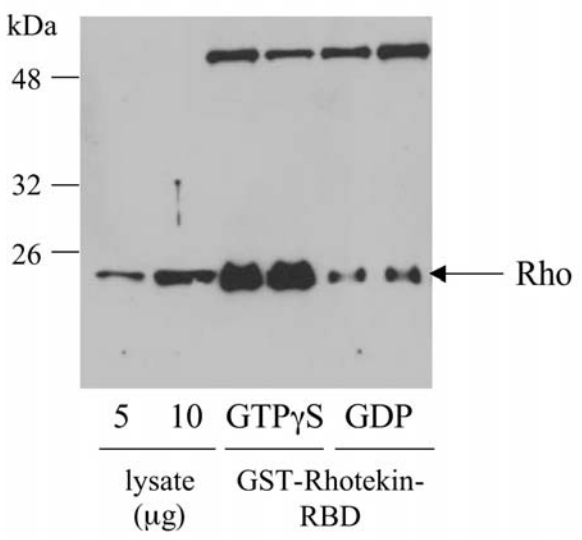

Figure 1. Western blot of control reactions. NIH3T3 cell lysates (500µg) were treated *in vitro* with GTPγS or GDP to activate or inactivate Rho (refer to optional step B). The lysates were then incubated with GST-Rhotekin-RBD and the Glutathione Resin. GTPγS-treated lysate was also incubated with GST alone in the presence of Glutathione Resin (negative control). Half of the volume of the eluted samples (25µL) and 10µg of cell lysate were separated by 12% SDS-PAGE, transferred to a nitrocellulose membrane and probed with Anti-Rho Antibody and then with HRP-conjugated secondary antibody. The detection was performed with SuperSignal West Pico Chemiluminescent Substrate (Product No. 34080) and exposure to X-ray film. An additional signal is detected at ~50kDa.

Related Thermo Scientific Products

21065	**Pierce Background Eliminator Kit,** for eliminating background from overexposed X-ray film
28320	**Surfact-Amps® 20 (Active Ingredient: Tween-20)**, 6 × 10mL
32460	**Stabilized Goat Anti-Rabbit IgG (H+L), Peroxidase Conjugated**, 2mL
34090	**CL-XPosure™ Film (5" × 7" sheets)**, 100 sheets/pkg
21059	**Restore® Western Blot Stripping Buffer,** 500mL

General References

Ren, X-D. and Schwartz, M.A. (2000). Determination of GTP loading on Rho. *Methods. Enzymol.* **325:**264-272.

Ren, X-D., *et al.* (1999). Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. *EMBO J.* **18(3):**578-585.

Reid, T., *et al.* (1996). Rhotekin, a new putative target for Rho bearing homology to Serine/Threonine kinase, PKN, and Rhophilin in the Rho-binding domain. *J. Biol.Chem.* **271(23):**13556-60.