

Active Cdc42 Pull-Down and Detection Kit

16119

2274.2

Number	Description
16119	Active Cdc42 Pull-Down and Detection Kit , contains sufficient reagents for 30 pull-down reactions

Kit Contents:**Box 16119X (these items ship together on dry ice; upon receipt store at -20°C):**

GST-Pak1-PBD, 600µg, contains 1-2mg/L in 25mM Tris•HCl, pH 7.2, 150mM NaCl and 10% glycerol; ~35kDa; GST-Pak1-PBD interacts with Cdc42 from human and mouse, and possibly from all mammalian species, store at -20°C

Anti-Cdc42 Antibody, 300µL (5 units), mouse monoclonal IgG1; Anti-Cdc42 antibody reacts with Cdc42 of human, dog, rat and mouse; store at -20°C. Note: One unit of Anti-Cdc42 antibody is defined as the amount of antibody required to detect Cdc42 in 40µg NIH3T3 whole cell lysate by Western blotting (8.5 × 7.5cm membrane).

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

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

Box 16119Y (these items ship together with an ice pack; upon receipt store at 4°C):

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 MgCl₂, 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 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 Cdc42 Pull-Down and Detection Kit is a simple and fast tool to monitor Cdc42 small GTPase activation. The kit provides a GST-fusion protein containing the p21-binding domain (PBD) of p21-activated kinase 1 (Pak1) along with glutathione agarose resin to specifically pull down active Cdc42 and an anti-Cdc42 antibody for Western blot detection. Also included are two control nucleotides, GTP γ S and GDP, which can be used to generate positive and negative control lysates, respectively. Each kit is functionally tested to ensure component performance.

GTPase Cdc42 (~22kDa) regulates the organization of the actin cytoskeleton and gene transcription. Activation of Cdc42 promotes actin polymerization to form filopodia or microspikes and is associated with integrin complexes. Furthermore, Cdc42 has been shown to activate Rac, such that filopodial extensions are usually seen associated with lamellipodial protrusions. Like other small GTPases, Cdc42 is active when bound to GTP and inactive when bound to GDP.

Important Product Information

- Cdc42-GTP is quickly hydrolyzed to Cdc42-GDP; use fresh lysate for each assay.
- For optimal pilot experiments, use from 500 μ g to 1mg 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 Bradford Protein Assay.
- For best results, always use protease inhibitors for cell lysis and keep lysates on ice between steps.
- For best results when performing the Western blotting procedure, use Pierce[®] Goat Anti-Mouse IgG (H+L), Peroxidase Conjugated (Product No. 31430) and Thermo Scientific SuperSignal West Pico Chemiluminescent Substrate (Product No. 34080)(refer to Additional Information Section, Figure 1). If 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 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-Mouse IgG-Horseradish Peroxidase Conjugate (Product No. 31430)
- 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 MgCl₂
- Sodium azide (NaN₃)
- Electrophoresis Apparatus
- Variable-speed Bench-top Microcentrifuge

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 75cm² 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 75cm² flask (approx. 1-2 × 10⁷ cells) at 100 × *g* for 5 minutes and then resuspend cells in 10mL of 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 MgCl₂ (for a final concentration of 60mM) and vortex the sample.

C. Affinity Precipitation of Activated Cdc42

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 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 6000 × *g* for 10-30 seconds. Discard the flow-through.
5. Thaw the GST-Pak1-PBD on ice and immediately make 20μg aliquots. Store aliquots for later use at -70°C.
6. Add 20μg of GST-Pak1-PBD 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.
18. 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 on SDS-PAGE and transfer to nitrocellulose or PVDF membrane.
 2. Block the membrane in TBS containing 3% BSA at room temperature for 1-2 hours.
 3. Rinse membrane with TBS containing 0.05% Tween-20 Detergent (TBST) for 5 minutes.
 4. Prepare a solution containing the Anti-Cdc42 mouse monoclonal antibody (1:167 dilution) in 3% BSA and 0.1% NaN₃ in TBST. An example of a 1:167 dilution is to add 60µL of the stock antibody solution to 10mL of buffer.
 5. Incubate the membrane in the anti-Cdc42 antibody solution at 4°C overnight.
Note: If the number of pull-down reactions per blot is low, the diluted anti-Cdc42 antibody solution can be re-used up to three times with no performance loss. Store the diluted anti-Cdc42 antibody solution at 4°C for up to two months.
 6. Wash membrane five times for 5 minutes each with TBST.
 7. Dilute the anti-mouse IgG-HRP-conjugate in TBST containing 5% nonfat dry milk [e.g., if using Pierce Goat Anti-Mouse IgG (H+L), Peroxidase Conjugated (Product No. 31430), dilute at 1:20,000 to 1:100,000].
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 on the Western blot.
 8. Incubate the membrane in the diluted anti-mouse IgG HRP Conjugate at room temperature for 1 hour.
 9. Wash membrane five times for 5 minutes each with TBST.
 10. Incubate the membrane with chemiluminescent substrate (e.g., SuperSignal West Pico Chemiluminescent Substrate).
 11. Immediately expose the membrane to X-ray film or a CCD camera.
Note: The Cdc42 band is located at ~24kDa.

Troubleshooting

Problem	Cause	Solution
No activated Cdc42 detected	Primary antibody required optimization	Optimize the primary antibody concentration
	Incorrect secondary antibody used for detection	Use goat anti-mouse IgG
	No activated Cdc42 was present in lysates	Include GTP γ S-treated lysate as positive control for pull-down
	Insufficient activated Cdc42	Increase the amount of lysate used for detection
	GST-Pak1-PBD was not added	Add GST-Pak1-PBD to the reactions
	Degraded GST-Pak1-PBD	Avoid multiple freeze/thaw cycles of GST-Pak1-PBD
	Degraded proteins	Add protease inhibitors to the Lysis/Binding/Wash Buffer before lysing cells
Detection system was not functioning properly or required optimization	Consult the instructions for the detection system being used	
No signal with GTP γ S or strong signal with GDP	GTP γ S or GDP were no longer functional	Aliquot GTP γ S or GDP after the first thaw and store at -20°C; avoid repeated freeze/thaw cycles of the resuspended solution
	Incorrect concentration of EDTA or MgCl ₂	Prepare new solutions with correct concentration
Western blot resulted in high background	Inadequate blocking and/or washing	Consult the instructions for the detection system being used
	Secondary antibody concentration was too high	

Additional Information

Cdc42 is active when bound to GTP and inactive when bound to GDP. Active Cdc42 binds specifically to the p21-binding domain (PBD) of p21-activated kinase 1 (Pak1), leading its activation. Therefore, the PBD of Pak1 can be used as a probe to specifically isolate the active form of Cdc42 (Figure 1).

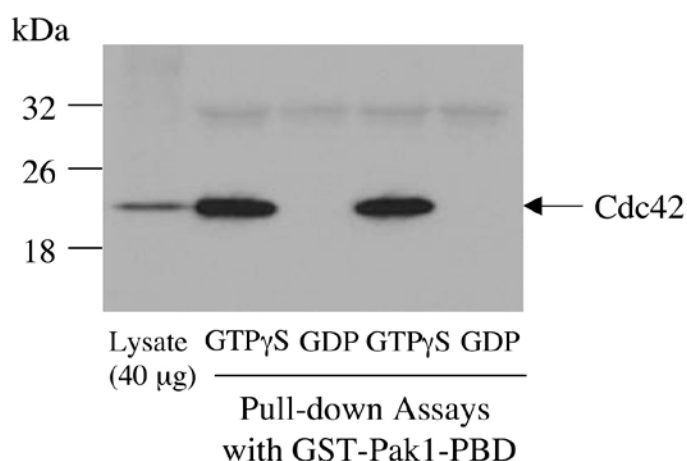


Figure 1. Western blot of control reactions. NIH3T3 cell lysates (500µg) were treated *in vitro* with GTP γ S or GDP to activate or inactivate Cdc42 (refer to optional step B). The lysates were then incubated with 20µg of GST-Pak1-PBD and the Glutathione Resin. Lysate (40µg) and half the volume of eluted samples (25µL) were separated by 4-20% SDS-PAGE, transferred to a nitrocellulose membrane and probed with Anti-Cdc42 Antibody. Pierce Goat Anti-Mouse IgG (H+L), Peroxidase Conjugated (Product No. 31430; 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. The exposure time was 2 seconds.

Related Thermo Scientific Products

25200-25244	Precise™ Protein Gels (see catalog or website for a complete listing)
21065	Pierce Background Eliminator Kit , for eliminating background from overexposed X-ray film
23236	Pierce Coomassie Plus (Bradford) Protein Assay Reagent
23227	BCA Protein Assay Reagent Kit
22660	Pierce 660nm Protein Assay Reagent , 750mL
28320	Surfact-Amps® 20 Detergent Solution (Active Ingredient: Tween-20 Detergent) , 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
31430	Pierce Goat Anti-Mouse IgG (H+L), Peroxidase Conjugated , 2mL
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 , 7.7mg 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

Benard, V. and Bokoch, G. (2002). Assay of Cdc42, Rac, and Rho GTPase activation by affinity methods. *Methods Enzymol* **345**, 349-359.

Burbelo, P.D., Drechsel, D. and Hall A. (1995). A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases. *J Biol Chem* **270**, 29071.

Benard, V., Bohl, B. and Bokoch, G. (1999). Characterization of Rac and Cdc42 activation in chemoattractant-stimulated human neutrophils using a novel assay for active GTPases. *J Biol Chem* **274(19)**, 13198-204

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