INSTRUCTIONS

Active Ras Pull-Down and Detection Kit

16117 2268.0

Number	Description
16117	**Active Ras Pull-Down and Detection Kit,** contains sufficient reagents for 30 pull-down reactions

Kit Contents:

Box 89855X (these items ship together on dry ice; upon receipt store at -70°C):

GST-Raf1-RBD, 2.4mg, contains 2-3mg/mL in 50mM Tris•HCl, pH 7.2, 150mM NaCl, 0.5% Triton® X-100, 5mM $MgCl_2$, 1mM DTT and 10% glycerol; GST-Raf1-RBD interacts with Ras from human and mouse, and possibly from 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 89855Y (these items ship together with an ice pack; upon receipt store at 4°C):

Anti-Ras Antibody, 250µL (5 units) mouse monoclonal $Ig_{2a}κ$; Anti-Ras antibody reacts with Ras of human, rat, and mouse; store at 4°C. Note: One unit of Anti-Ras antibody is defined as the amount of antibody required to efficiently detect Ras in 20µg NIH3T3 whole cell lysate by Western blotting (8.5 × 7.5cm membrane).

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

1X Lysis/Binding/Wash Buffer, 100mL, contains 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, contains 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 Active Ras Pull-Down and Detection Kit is a simple and fast tool to monitor Ras small GTPase activation. The kit provides a GST-fusion protein of the Ras-binding domain (RBD) of Raf1 along with glutathione agarose resin to specifically pull down active Ras and an anti-Ras 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. Ras (~21kDa) is a key regulator of cell growth and is implicated in tumorigenesis, tumor invasion and morphogenesis. It has been estimated that 30% of human tumors contain an activating mutation in Ras. Like other small GTPases, Ras is active when bound to GTP and inactive when bound to GDP.

Important Product Information

- Ras-GTP is quickly hydrolyzed to Ras-GDP; use fresh lysate for each 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 and keep lysates on ice between steps.
- For optimal pilot experiments, use 500µg to 1mg of total lysate per assay.
- 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 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 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 (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 $MgCl_2$
- Sodium azide (NaN_3)
- Electrophoresis Apparatus
- Variable-speed Bench-top Microcentrifuge

Procedure for using the Ras 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 Ras

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 6,000 × *g* for 10-30 seconds. Discard the flow-through.
5. Thaw the GST-Raf1-RBD on ice and immediately make 80µg aliquots. Store aliquots for later use at -70°C.
6. Add 80µg of GST-Raf1-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% 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 or PVDF membrane.
2. Block membrane in TBS containing 3% BSA at room temperature for 1-2 hours.
3. Rinse membrane with TBS containing 0.05% Tween-20 (TBST) for 5 minutes.
4. Prepare a solution containing the Anti-Ras Antibody (1:200 dilution) in 3% BSA and 0.1% NaN_3 in TBST. An example of a 1:200 dilution is to add 50µL of the stock antibody solution to 10mL of buffer.
5. Incubate the membrane in the primary anti-Ras antibody solution at 4°C overnight.

   Note: If the number of pull-down reactions per blot is low, the diluted anti-Ras antibody solution can be re-used up to three times with no performance loss. Store the diluted anti-Ras 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-mouse IgG-HRP-conjugate in TBST containing 5% nonfat dry milk [e.g., if using Pierce Goat Anti-Mouse IgG (H+L), Peroxidase Conjugated dilute within the range of 1:20,000 to 1:100,000].

   Note: Ensure that the nonfat dry milk is completely dissolved in TBST (e.g. mix 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 membrane in the anti-mouse IgG-HRP Conjugate solution at room temperature for 1 hour.
9. Wash the membrane five times for 5 minutes each with TBST.
10. Incubate 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 Ras band is located at ~21kDa.

Troubleshooting

Problem	Cause	Solution
No activated Ras detected	Primary antibody requires optimization	Optimize the primary antibody concentration
	Incorrect secondary antibody used for detection	Use goat anti-mouse IgG
	No activated Ras present in lysates	Include GTPγS-treated lysate as positive control for pull-down
	Insufficient activated Ras	Increase the amount of lysate used for detection
	GST-Raf1-RBD was not added	Add GST-Raf1-RBD to the reactions
	Degraded GST-Raf1-RBD	Avoid multiple freeze/thaw cycles of GST-Raf1-RBD
	Degraded proteins	Add protease inhibitors to the Lysis/Binding/Wash Buffer before lysing the cells
	Detection system is 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 are no longer functional	Aliquot GTPγS or GDP after the first thaw and store at -70°C; avoid repeated freeze/thaw cycles
	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 is too high	

Additional Information

Ras is active when bound to GTP and inactive when bound to GDP. Active Ras binds specifically to Ras-binding domain (RBD) of Raf1, leading its activation. Therefore, the RBD of Raf1 can be used as a probe to specifically isolate the active form of Ras (Figure 1).

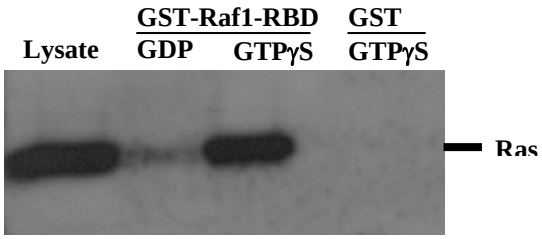

Figure 1. Western blot of control reactions. NIH 3T3 cell lysates (500µg) were treated *in vitro* with GTPγS or GDP to activate or inactivate Ras (refer to optional step B). The lysates were then incubated with GST-Raf1-RBD and the Glutathione Resin. GTPγS-treated lysate was also incubated with GST alone in the presence of Glutathione Resin (negative control). Fifty percent of the eluted samples (25µL) and 10µg of cell lysate were separated by 4-20% SDS-PAGE, transferred to a nitrocellulose membrane and probed with Anti-Ras 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.

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 (Active Ingredient: Tween®-20)**, 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

Chiu, V.K., *et al.* (2002). Ras signaling on the endoplasmic reticulum and the Golgi. *Nat Cell Biol.* **4(5):**343-50.

Feig, L.A. and Buchsbaum, R.J. (2002). Cell signaling: life or death decisions of Ras proteins. *Curr Biol.* **12(7):**R259-61.

Taylor, S.J., *et al.* (2001). Nonradioactive Determination of Ras-GTP levels using activated Ras interaction assay. *Methods. Enzymol.* **333:**333-48.