Draft.

Rhotekin Rho Binding Domain

(recombinant protein expressed in *E. coli*)
Catalog # 14-383
Lot # 20171

Product Description: A GST-tagged fusion protein, corresponding to residues 7-89 of mouse Rhotekin Rho Binding Domain, expressed in *E.* coli. Provided bound to glutathione agarose. Purity > 90%.

Specificity: Specifically binds to and precipitates GTP-Rho, not GDP-Rho from cell lysates.

Formulation: 650µg of GST-tagged Rhotekin Rho Binding Domain, in **1 ml** glutathione-agarose slurry of 50 mM Tris, pH 7.5, 0.5% Triton X-100, 150 mM NaCl, 5mM $MgCl_2$, 1mM DTT, 1µg/ml each of aprotinin and leupeptin, 0.1 mM PMSF, and 10% glycerol. Liquid at –70°C.

Storage and Stability: Stable for 6 months at -70°C from date of shipment. Mix the agarose slurry well before doing affinity precipitation of GTP-Rho.

FOR RESEARCH USE ONLY
NOT FOR USE IN HUMANS

Quality Control Testing: 32 µg (or50µl) of GST-tagged Rhotekin Rho Binding Domain precipitated GTP-Rho from 3T3 cell lysates that had been loaded with GTPγS or GDP. The precipitated GTP-Rho was detected by Western immunoblot analysis using anti-Rho (Cat. # 06-770) (2µg/ml).

Affinity Precipitation/Immunoblot Protocol

Stock Solutions:

Lysis Buffer: 50mM Tris, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500mM NaCl, 10mM $MgCl_2$, 10µg/ml each of aprotinin and leupeptin, and 1mM PMSF.

Wash Buffer: 50mM Tris, pH 7.2, 1% Triton X-100, 150mM NaCl, 10mM $MgCl_2$, 10µg/ml each of aprotinin and leupeptin, and 0.1mM PMSF.

100X GTPγS, 10mM: Catalog # 20-176. Use 10µl of GTPγS for GTP-labeling of 1ml of cell lysate.

100X GDP, 100mM: Catalog # 20-177. Use 10µl of GDP for GDP-labeling of 1ml of cell lysate.

In vitro GTPγS/GDP Protein Loading for positive and negative controls:

1. Place 0.5ml of cell lysate into two microcentrifuge tubes.
2. To each tube, add 1µl of 0.5M EDTA (final concentration 1mM) and 5µl of 100X GTPγS, 10mM (final concentration 100µM) to one tube for a positive control and 5µl of 100X GDP, 100mM (final concentration 1mM) to the second tube as a negative control.
3. Incubate the tubes at 30°C for 15 minutes with agitation.
4. Terminate the reaction by placing the tube on ice and adding $MgCl_2$ (final concentration 60mM).

Assay Procedure:

Note: While Upstate recommends using fresh lysates because GTP-Rho is quickly hydrolyzed to GDP-Rho, frozen lysates may be used provided that the lysates were snap frozen in liquid nitrogen and stored at or below -70°C. Add Rhotekin Rho Binding Domain agarose directly to the lysate immediately after removing cellular debris and insoluble material by centrifugation.

1. Grow cells until they are approximately 75-80% confluent.
2. Lyse cells with 1ml of lysis buffer per 150mm tissue culture plate.
3. Immediately add 20-32µg (30-50µl) of Rhotekin Rho Binding Domain agarose per 0.5ml of cell lysate. Note that the optimal conditions for each experimental system should be determined empirically.
4. Gently rock the reaction mixture at 4°C for 45 minutes.
5. Collect the agarose beads by pulsing for 5 seconds in the microcentrifuge at 14,000 x g, remove the supernatant. Wash the beads 3 times with 0.5ml of wash buffer.
6. Resuspend the agarose beads in 30µl of 2X Laemmli sample buffer containing 40mM DTT and boil for 5 minutes.
8. Perform SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on the entire boiled bead slurry and transfer the proteins to nitrocellulose. Wash the blotted nitrocellulose twice with water.

Assay Procedure:

9. Block the blotted nitrocellulose in freshly prepared PBS containing 3% nonfat dry milk (PBS-MLK) for 30 minutes at room temperature with constant agitation.
10. Incubate the nitrocellulose with 2µg/ml of anti-Rho (Catalog # 06-770) diluted in freshly prepared PBS-MLK overnight with agitation at 4°C.
11. Wash the nitrocellulose twice with water.
12. Incubate the nitrocellulose in the secondary reagent of choice (a goat anti-rabbit HRP conjugated IgG, 1:3000 dilution was used) in PBS-MLK for 1.5 hours at room temperature with agitation.
13. Wash the nitrocellulose with water twice.
14. Wash the nitrocellulose in PBS containing 0.05% Tween 20 for 15-30 minutes.
15. Rinse the nitrocellulose with water.
16. Use detection method of choice (enhanced chemiluminescence was used).