V. 1.1

Rhotekin-RBD Protein
(Human Recombinant)
Cat. # RT01

Upon arrival store at 4°C (desiccated)
See datasheet for storage after reconstitution

Material

The Rho-GTP binding domain (RBD) of the human Rhotekin protein has been overexpressed and purified as a GST-tagged recombinant protein in *E. coli*. The Rhotekin-RBD consists of amino acids 7-89 of the Rhotekin protein. When tagged with GST (28 kDa) the recombinant protein has an approximate molecular weight of 35 kDa. The protein is supplied as 500 µg of lyophilized protein.

Storage and Reconstitution

Reconstitute in 250 µl of Milli-Q water to give a 2.0 mg/ml protein solution. When reconstituted, the protein will be in the following buffer: 25 mM Tris pH 7.5, 25 mM NaCl, 0.5 mm $MgCl_2$, 2.5% sucrose and 0.5% dextran. For storage, the protein should be aliquoted into "experiment sized" amounts, snap frozen in liquid nitrogen and stored at -70°C. Under these conditions the Rhotekin-RBD protein is stable for 6 months. The protein MUST NOT be exposed to repeated freeze thaw cycles.

Purity

Protein purity is determined by scanning densitometry of Coomassie Blue stained protein on a 12% SDS polyacrylamide gel. Rhotekin-RBD protein was determined to be approx. 90% pure (see Figure 1).

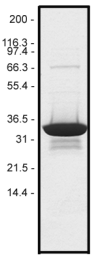

Figure 1. Rhotekin-RBD Protein Purity Determination. A 20 µg sample of recombinant GST-Rhotekin-RBD (molecular weight approx. 35 kDa) was separated by electrophoresis in a 12% SDS-PAGE system and stained with Coomassie Blue. Protein quantitation was performed using the Precision Red™ Protein Assay Reagent (Cat. # ADV02). Mark12 molecular weight markers are from Invitrogen.

Biological Activity Assay

Rhotekin-RBD specifically recognizes and binds to the active "GTP-bound" form of Rho protein (1, 2). It has a much lower affinity for the inactive "GDP-bound" form of Rho. Biological activity of GST-tagged Rho-RBD is therefore determined by its selectivity for GTP-bound Rho in a pull-down assay using a human platelet extract loaded with either GTPγS or GDP. It should be noted that in an *in vivo* Rho activation assay you will only get 1 - 5% Rho activation even when the cell lysate is loaded with GTPγS. This is in agreement with the published figures for Rho activation (3).

Reagents

1. GST-tagged Rhotekin-RBD protein (Cat. # RT01)
2. Glutathione bead slurry (Amersham Biosciences, Cat. # 27-4574-01)
3. Rhotekin-RBD beads (Cat. # RT02)
4. Loading Buffer (150 mM EDTA)
5. STOP Buffer (600 mM $MgCl_2$)
6. Wash Buffer (25 mM Tris pH 7.5, 30 mM $MgCl_2$, 40 mM NaCl)
7. Cell Lysis Buffer (50 mM Tris pH 7.5, 10 mM $MgCl_2$, 0.3 M NaCl, 2% IGEPAL)
8. GTPγS (20 mM solution)
9. GDP (100 mM solution)
10. Human platelet extract (20 mg/ml) prepared in 50 mM PIPES pH 7.0,130 mM NaCl, 1 mM PMSF, 1 mM DTT, 5 µg/ml leupeptin, 0.5% Triton X -100
11. Protease inhibitor cocktail, 100x (Cat. # PIC02)
12. Anti-Rho monoclonal antibody (Cat. # ARH03)

Equipment

1. Micro-centrifuge at 4°C
2. SDS-PAGE and Western blot apparatus

Method

1. Dilute platelet extract to 0.5 mg/ml in Cell Lysis Buffer plus 1x protease inhibitor cocktail.
2. Centrifuge at 16,000 x g at 4°C for 15 min to pellet cell membranes and insoluble material.
3. Divide the supernatant equally between four tubes labeled 1-4.
4. Add $1/10^{th}$ the volume of Loading Buffer to each tube (final conc. 15 mM).
5. Add $1/100^{th}$ the volume of GDP to tubes 1 and 3 (final conc. 1.0 mM).
6. Add $1/100^{th}$ the volume of GTPγS to tubes 2 and 4 (final conc. 0.2 mM).
7. Incubate all tubes at room temperature for 15 min.
8. Stop the reaction by adding $1/10^{th}$ the volume of STOP Buffer to all tubes (final conc. 60 mM).
9. Resuspend Rhotekin-RBD protein and add 100 µg (40 µl) protein to tubes 1 and 2. Note: Titration of the amount of Rhotekin-RBD (50-200 µg) to add per reaction is recommended.
10. Resuspend the Rhotekin-RBD beads (Cat. # RT02) and add 100 µg to tubes 3 and 4.
11. Gently rotate all tubes at 4°C for 30 min.
12. Add 30 µl of a 50% slurry of glutathione beads to tubes 1 and 2. Add 30 µl of Lysis Buffer to tubes 3 and 4.
13. Gently rotate all tubes at 4°C for 30 min.

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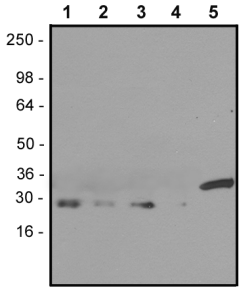
14. Pellet the beads by centrifugation at 2000 x g, 4°C for 1 min.
15. Remove the supernatant and wash the beads twice in 500 µl of Wash Buffer.
16. Pellet the beads by centrifugation and resuspend the beads from each tube in 30 µl of SDS sample buffer.
17. The bead and supernatant samples can now be analyzed by Western blot procedure using a RhoA specific mono-clonal antibody (Cat. # ARH03).
18. Typical assay results are shown in Figure 2.

Figure 2. Selective Binding of GST Tagged Rhotekin-RBD to the GTP-bound Form of Rho *In Vitro*. Human platelet extracts were loaded with either GTPγS (lanes 1 and 3), or GDP (lanes 2 and 4) as described in the method. The loaded extracts were incubated with either 100 µg of GST-tagged Rhotekin-RBD or 100 µg of Rhotekin-RBD pre-coupled to glutathione beads (Cat. # RT02). Protein-bead complexed samples were then subjected to Western blot analysis using a RhoA specific monoclonal antibody. Lane 5 shows 20 ng of recombinant His-RhoA control protein (Note: His-RhoA [30 kDa] runs slightly higher than endogenous RhoA [22 kDa] due to the presence of the His-tag). SeeBlue™ molecular weight markers are from Invitrogen.



Product Uses

- Measurement of the GTP/GDP ratio of RhoA *in vitro*.
- Quantitation of GTP-bound Rho from tissue culture cell lysates.

References

1. Aspenstrom, P. (1999). *Curr. Opin. In Cell Biol.* **11**: 95-102.
2. Ren, X.D., Kiosses, W.B., and Schwartz, M.A. (1999). *EMBO J.* **18**: 578-585.
3. Wei, Q and Adelstein, S. (2002). *Mol. Biol of the Cell.* **13**: 683-697.

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