V. 1.0

Rhotekin-RBD Protein GST Beads
(Human Recombinant)
Cat. # RT02

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 as a GST-tagged recombinant protein in E. coli. The Rhotekin-RBD contains amino acids 7-89 of the Rhotekin protein, and has an approximate molecular weight of 35 kDa. The protein is supplied as 2 mg of lyophilized bead bound protein. The bead matrix is dark purple in color for easy detection.

Storage and Reconstitution
Reconstitute in 600 µl of distilled water to give a 3.3 mg/ml bead bound protein solution. When reconstituted the bead bound protein will be in the following buffer: 40 mM Tris pH 7.5, 40 mM NaCl, 1.5% dextran and 8% sucrose. For storage, the beads should be aliquoted into "experiment sized" amounts, snap frozen in liquid nitrogen and stored at -70°C. Under these conditions the Rhotekin-RBD beads are stable for 6 months. We recommend 60 µg (18 µl) sized aliquots for each activation reaction (see Biological Activity Assay in this protocol). The amount of Rhotekin RBD-beads can be titrated (25 -100 µg) to determine the optimal amount to use for each experimental protocol. 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 was determined to be approx. 90% pure (see Figure 1).

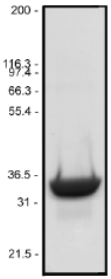

Figure 1. ***Rhotekin-RBD GST Tagged Protein Purity Determination.*** A 20 µg sample of recombinant Rhotekin-RBD GST-tagged protein (molecular weight approx. 35 kDa) was separated by electrophoresis in a 12% SDS-PAGE system and stained with Coomassie Blue. Protein quantitation was performed using the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

Biological Activity Assays
The Rhotekin-RBD protein specifically recognizes and binds to the active "GTP-bound" form of Rho protein (1, 2) and has a much lower affinity for the inactive "GDP-bound" form of Rho. Biological activity of Rho-RBD protein is therefore determined by its selectivity for GTP-Rho protein.

Assay #1: Artificially Loaded Platelet Extracts
A standard assay for determining the activity of the beads comprises 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 beads (Cat. # RT02)
2. Loading Buffer (150 mM EDTA)
3. STOP Buffer (600 mM $MgCl_2$)
4. Wash Buffer (25 mM Tris pH 7.5, 30 mM $MgCl_2$, 40 mM NaCl)
5. Cell Lysis Buffer (50 mM Tris pH 7.5, 10 mM $MgCl_2$, 0.3 M NaCl, 2% IGEPAL)
6. GTPγS (20 mM solution)
7. GDP (100 mM solution)
8. 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
9. Protease inhibitor cocktail, 100x (Cat. # PIC02)
10. Anti-Rho monoclonal antibody (Cat. # ARH03)

Note: All components except for the platelet extract can be found in the RhoA Activation Assay Kit (Cat. # BK036)

Equipment
1. Microfuge 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 14k rpm at 4°C for 15 min to pellet cell membranes and insoluble material.
3. Divide the supernatant equally between two tubes.
4. Add 1/10th the volume of Loading Buffer to each tube (final conc. 15 mM).
5. Add 1/100th the volume of GDP to one tube (final conc. 1.0 mM).
6. Add 1/100th the volume of GTPγS to the other tube (final conc. 0.2 mM).

Phone: (303) 322.2254 **Fax:** (303) 322.2257
Customer Service: cserve@cytoskeleton.com
Technical Support: tservice@cytoskeleton.com

7. Incubate both tubes at room temperature for 15 min.
8. Stop the reaction by adding 1/10th the volume of STOP Buffer to each tube (final conc. 60 mM).
9. Resuspend Rhotekin-RBD protein beads and add 60 µg (20 µl) protein bound beads to each reaction tube.
10. Gently rotate the tubes at 4°C for 1 h.
11. Pellet the beads by centrifugation at 5k rpm, 4°C for 1 min.
12. Remove the supernatant and wash the beads twice in 500 µl of Wash Buffer.
13. Pellet the beads by centrifugation and resuspend the beads from each tube in 30 µl of SDS sample buffer.
14. The bead and supernatant samples can now be analyzed by Western blot procedure using a RhoA specific monoclonal antibody (Cat. # ARH03).
15. Typical assay results are shown in Figure 2.

Figure 2. Selective Binding of Rhotekin-RBD GST Tagged Protein Beads to the GTP-bound Form of Rho In Vitro.

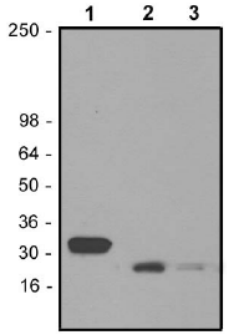

Figure 2 Legend. Human platelet extracts were loaded with either GTPγS (lane 2), or GDP (lane 3) as described in the method. The loaded proteins were incubated with 60 µg of Rhotekin-RBD GST tagged protein beads, separated by centrifugation, and subjected to Western blot analysis using a RhoA specific monoclonal antibody. Lane 1 shows 50 ng of recombinant His-RhoA control protein (Note: His-RhoA (30 kDa) runs slightly higher than endogenous RhoA (23 kDa) due to the presence of the His-tag). See Blue molecular weight markers are from Invitrogen

Assay #2: Calpeptin Activation of RhoA in Swiss 3T3 Cells

Figure 3. Selective Binding of Rhotekin-RBD GST Tagged Protein Beads to calpeptin activated RhoA in Swiss 3T3 cells.

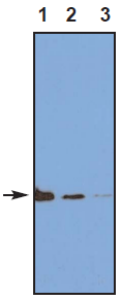

Figure 3 Legend. Swiss 3T3 cells were grown to 40% confluency in DMEM plus 10% FCS (Fetal Calf Serum) before being grown for 24h in DMEM plus 0.5% FCS and then serum starved by growing in DMEM only for 24h. Cells were either harvested straight after serum starvation or treated for 20 minutes with calpeptin (0.1 mg/ml) before harvesting. Cells were harvested in Cell Lysis Buffer (50 mM Tris pH 7.5, 10 mM $MgCl_2$, 0.3 M NaCl, 2% IGEPAL).

A rhotekin-RBD pull down assay was carried out as described in Assay #1,steps 9-15, except that 50 µg of rhotekin-RBD beads were used per assay. Lane 1 shows 40 µg total cell lysate, Lane 2 shows pull down results from 500 µg of the calpeptin treated cell lysate, Lane 3 shows pull down results from 500 µg of the serum starved cell lysate.

Arrow indicates endogenous RhoA signal (23kD).

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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