V. 1.0

**Raf-RBD Protein GST Beads**
**(Human recombinant)**
**Cat. # RF02**

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

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**Material**

The Ras GTP Binding Domain (RBD) of human Raf protein kinase has been produced in a bacterial expression system. The recombinant protein consists of amino acids 51-149 of Raf kinase and is tagged with GST (28 kDa) at its amino terminus. The approximate molecular weight of the Raf-RBD GST protein is 35 kDa. The protein is supplied as 2 mg of lyophilized bead bound protein. The protein 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 (10 µl of bead slurry is sufficient for 1 assay), snap frozen in liquid nitrogen and stored at -70°C. Under these conditions the Raf-RBD beads are stable for at 6 months. We recommend 30 µg (10 µl) sized aliquots for each activation reaction (see Biological Activity Assay in this 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. Raf-RBD protein was determined to be approx. 85% pure (see Figure 1).

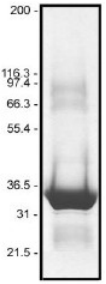


**Figure 1. Raf-RBD GST Protein Purity Determination.** A 20 µg sample of recombinant Raf-RBD GST protein (molecular weight approx. 35 kDa) was separated by electrophoresis in a 12% SDS-PAGE system and stained with Coomassie Blue. Protein quantitation was performed using the Precision Red Protein Assay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

**Biological Activity Assay**

The Raf-RBD protein specifically recognizes and binds to the active "GTP-bound" form of Ras. (1, 2, 3, 4). It has a much lower affinity for the inactive "GDP-bound" form of Ras. Biological activity of Raf-RBD protein is therefore determined by its selectivity for GTP-Ras. A standard assay for Raf-RBD protein beads is a Ras protein pulldown assay from bovine brain extracts loaded with GTPγS or GDP.

**Reagents**

1. GST-tagged Raf-RBD protein beads (Cat. # RF02)
2. Recombinant Ras-GST protein (Cat. # RS01)
3. Loading buffer (150 mM EDTA)
4. Stop buffer (600 mM $MgCl_2$)
5. Wash buffer (25 mM Tris pH 7.5, 30 mM $MgCl_2$, 40 mM NaCl)
6. Cell lysis buffer (50 mM Tris pH 7.5, 10 mM $MgCl_2$, 0.3 M NaCl, 2% IGEPAL)
7. GTPγS (20 mM solution)
8. GDP (100 mM solution)
9. Bovine brain extract (20 mg/ml) prepared in 50 mM PIPES pH 7,130 mM NaCl, 1 mM PMSF, 1 mM DTT, 5 ug/ml leupeptin, 0.5% Triton X-100
10. Protease inhibitor cocktail (Cat. # PIC02)
11. Pas Ras monoclonal antibody

**Equipment**

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

**Method**

1. Dilute 100 µl of bovine brain extract to 2 mg/ml by adding 900 µl of lysis buffer plus protease inhibitors.
2. Centrifuge at 14k rpm at 4°C for 15 min to pellet cell membranes and insoluble material.
3. Divide the remaining supernatant equally between two tubes.
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 one tube (final conc. 1.0 mM).
6. Add 1/100th the volume of GTPγS to the other tube (final conc. 0.2 mM).
7. Incubate both tubes at room temperature for 15 min.
8. Stop the reaction by adding $1/10^{th}$ the volume of stop buffer to each tube (final conc. 60 mM).
9. Resuspend the Raf-RBD protein beads and add 30 µg (10 µl) protein bound beads to each reaction tube.
10. Gently rotate the tubes at 4°C for 1 h.


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11. Centrifuge out the beads at 5k rpm at 4°C for 1 min.
12. Remove the supernatant and wash the beads twice in 500 µl of wash buffer.
13. Centrifuge the beads and resuspend each tube in 20 µl of SDS sample buffer.
14. The bead and supernatant samples can now be analyzed by Western blot using a Pan Ras monoclonal antibody.
15. Typical assay results are shown in Figure 2.

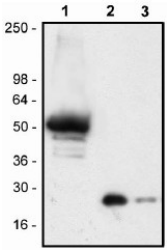


**Figure 2. Selective Binding of Raf-RBD Protein Beads to the GTP-bound Form of Ras *In Vitro*.** Bovine brain extracts were loaded with either GTPγS (lane 2), or GDP (lane 3) as described in the method. The loaded extracts were incubated with 30 µg of Raf-RBD GST protein beads, separated by centrifugation, and subjected to Western blot analysis using a Pan Ras monoclonal antibody. Lane 1 shows 50 ng of recombinant Ras-GST control protein (Note: Ras-GST runs at 50 kDa due to the presence of the GST tag). SeeBlue molecular weight markers are from Invitrogen

**Product Uses**

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

**References**

1. Van Aelst, L et al. (1993). *Proc Natl Acad Sci USA*. **90**: 6213-6217.
2. Vojtek, AB et al. (1993). *Cell*. **74**: 205-214.
3. Warne, PH. et al. (1993). *Nature*. **364**: 352-355.
4. Zhang, XF. et al. (1993). *Nature*. **364**: 303-313.

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