Active Ras Detection Kit

#8821

1 Kit
(30 immunoprecipitations)

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For Research Use Only. Not For Use In Diagnostic Procedures.

Species Cross-Reactivity: H, M

Description: The Active Ras Detection Kit provides all reagents necessary for measuring activation of Ras GTPase in the cell. GST-Raf1-RBD fusion protein is used to bind the activated form of GTP-bound Ras, which can then be immunoprecipitated with glutathione resin. Ras activation levels are then determined in western using a Ras Mouse mAb.

Specificity/Sensitivity: Active Ras Detection Kit detects endogenous levels of GTP-bound (active) Ras as shown in Figure 1. This kit detects proteins from the indicated species, as determined through in-house testing, but may also detect homologous proteins from other species.

Background: The Ras superfamily of small GTP-binding proteins (G proteins) comprise a large class of proteins (over 150 members) that can be classified into at least five families based on their sequence and functional similarities: Ras, Rho, Rab, Arf, and Ran (1-3). These small G proteins have both GDP/GTP-binding and GTPase activities and function as binary switches in diverse cellular and developmental events that include cell cycle preogression, cell survival, actin cytoskeletal organization, cell polarity and movement, and vesicular and nuclear transport (1). An upstream signal stimulates the dissociation of GDP from the GDP-bound form (inactive), which leads to the binding of GTP and formation of the GTP-bound form (active). The activated G protein then goes through a conformational change in its downstream effector-binding region, leading to the binding and regulation of downstream effectors. This activation can be switched off by the intrinsic GTPase activity, which hydrolyzes GTP to GDP and releases the downstream effectors. These intrinsic guanine nucleotide exchange and GTP hydrolysis activities of Ras superfamily proteins are also regulated by guanine nucleotide exchange factors (GEFs) that promote formation of the active GTP-bound form and GTPase activating proteins (GAPs) that return the GTPase to its GDP-bound inactive form (4).

The 21 kDa guanine-nucleotide binding proteins (K-Ras, H-Ras, and N-Ras) cycle between active (GTP-bound) and inactive (GDP-bound) forms (5). Receptor tyrosine kinases and G-protein-coupled receptors activate Ras, which then stimulates the Raf-MEK-MAPK pathway (6-8). GAP proteins normally facilitate the inactivation of Ras. However, in 30% of human tumors, point mutations in Ras prevent the GAP-mediated inhibition of this pathway (9). The most common oncogenic Ras mutation found in tumors is Gly12 to Asp (G12D), which prevents Ras inactivation, possibly by increasing the overall rigidity of the protein (9,10).

Components Ship As: 11871S	Item #	Kit Quantity	Storage Temp
GTPgS	11521	1 X 50 µl	–80°C
GDP	11522	1 X 50 µl	–80°C
GST-Raf1-RBD	8784	1 X 2.4 mg	–80°C
Components Ship As: 11860S	**Item #**	**Kit Quantity**	**Storage Temp**
Lysis/Binding/Wash Buffer	11524	1 X 100 mL	4°C
Glutathione Resin	11523	1 X 3 ml	4°C
SDS Sample Buffer	11525	1 X 1.5 ml	4°C
Spin Cup and Collection Tubes	11526	1 X 30 vial	RT
Components Ship As: 8832S	**Item #**	**Kit Quantity**	**Storage Temp**
Ras Mouse mAb	8832	1 X 250 µl	4°C

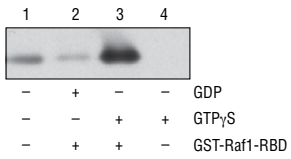

Figure 1. NIH/3T3 cell lysates (500 µl at 1 mg/ml) were treated in vitro with GTPγS or GDP to activate or inactivate Ras (refer to optional step C in protocol). The lysates were then incubated with glutathione resin and GST-Raf1-RBD (lanes 2 and 3). GTPγS-treated lysate was also incubated without GST-Raf1-RBD in the presence of glutathione resin as a negative control (lane 4). Western blot analysis of cell lysate (20 µg, lane 1) or 20 µl of the eluted samples (lanes 2, 3, and 4) was performed using a Ras mouse mAb. Anti-mouse IgG, HRP-linked Antibody #7076 was used as the secondary antibody.

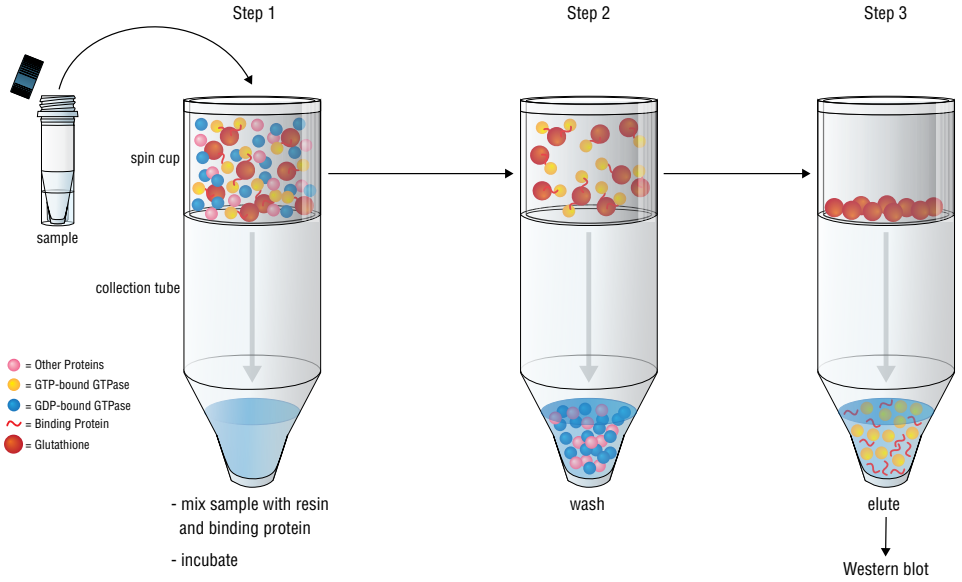

Figure 2. The GTP-bound GTPase pull-down process can be divided into 3 steps as shown. Step 1: Mix sample, binding protein, and glutathione resin in the spin cup and incubate at 4°C to allow GTP-bound GTPase binding to the glutathione resin through GST-linked binding protein. Step 2: Remove unbound proteins by centrifugation. Step 3: Elute glutathione resin-bound GTPase with SDS buffer. The eluted sample can then be analyzed by western blot.

Applications Key: **W**—Western **IP**—Immunoprecipitation **IHC**—Immunohistochemistry **ChIP**—Chromatin Immunoprecipitation **IF**—Immunofluorescence **F**—Flow cytometry **E-P**—ELISA-Peptide
Species Cross-Reactivity Key: **H**—human **M**—mouse **R**—rat **Hm**—hamster **Mk**—monkey **Mi**—mink **C**—chicken **Dm**—D. melanogaster **X**—Xenopus **Z**—zebrafish **B**—bovine **Dg**—dog **Pg**—pig **Sc**—S. cerevisiae **Ce**—C. elegans **Hr**—horse **All**—all species expected Species enclosed in parentheses are predicted to react based on 100% homology.

Background References:

(1) Takai, Y. et al. (2001) *Physiol Rev* 81, 153-208.

(2) Colicelli, J. (2004) *Sci STKE* 2004, RE13.

(3) Wennerberg, K. et al. (2005) *J Cell Sci* 118, 843-6.

(4) Vigil, D. et al. (2010) *Nat Rev Cancer* 10, 842-57.

(5) Boguski, M.S. and McCormick, F. (1993) *Nature* 366, 643-54.

(6) Avruch, J. et al. (1994) *Trends Biochem Sci* 19, 279-83.

(7) Buday, L. and Downward, J. (1993) *Cell* 73, 611-20.

(8) Huang, D.C. et al. (1993) *Mol Cell Biol* 13, 2420-31.

(9) Bos, J.L. (1989) *Cancer Res* 49, 4682-9.

(10) Ma, J. and Karplus, M. (1997) *J Mol Biol* 274, 114-31.

Active Ras Detection Kit Protocol

Additional Materials Required

Phenylmethanesulfonyl fluoride (PMSF) #8553
Blue Loading Buffer Pack #7722
Tris Buffered Saline with Tween® 20 (TBST-10X) #9997
Primary antibody dilution buffer: 1X TBST 5% BSA
Bovine Serum Albumin (BSA) #9998
Nonfat Dry Milk #9999
Color-coded Prestained Protein Marker, Broad Range (10-250 kDa) #74124
Biotinylated Protein Ladder Detection Pack #7727
Anti-mouse IgG, HRP-linked Antibody #7076
20X LumiGLO® Reagent and 20X Peroxide #7003
0.5 M EDTA, pH 8.0 #7011
1 M $MgCl_2$

A Solutions and Reagents

NOTE: Prepare solutions with ddH_2O or equivalently purified water.
1X Phosphate Buffered Saline (PBS)
1X Cell Lysis/Binding/Wash Buffer
NOTE: Add 1 mM PMSF immediately prior to use.

B Cell Lysis

1. To harvest cells under nondenaturing conditions, remove media and rinse cells once with ice-cold PBS. For non-adherent cells, pellet cells at 100xg for 5 min and resuspend cells in 10 ml ice-cold PBS.
2. Remove PBS and add 0.5 ml ice-cold 1X Lysis/Binding/Wash Buffer plus 1 mM PMSF to each plate (10 cm in diameter), or cell pellets from each 75 cm^2 flask (approx 1-2 x 10^7 cells).
3. Scrape cells off the plate and transfer to an appropriate tube. For non-adherent cells, just resuspend the pellet.
4. Votex the tube briefly and incubate on ice for 5 min.
5. Microcentrifuge at 16,000xg at 4°C for 15 min and transfer the supernatant to a new tube. The supernatant is the cell lysate. Fresh cell lysates are recommended for GTPase assays. Lysate protein concentration can be determined using BCA protein assay.

NOTE: We recommend making 1 mg/ml lysate in 1X Lysis/Binding/Wash Buffer for the following steps.

C *In vitro* GTPγS or GDP Treatment (Optional)

Perform the following treatments, GTPγS (positive control) and GDP (negative control), to ensure the immunoprecipitation procedures are working properly. Use 500 µg of cell lysate for each treatment. For best results, aliquot GTPγS and GDP at first use to minimize freeze/thaw cycles.

1. For 500 µl lysate, add 10 µl 0.5 M EDTA pH 8.0 (for a final concentration of 10 mM), vortex the sample.
2. Add 5 µl of 10 mM GTPγS (for a final concentration of 0.1 mM) or 5 µl 100 mM GDP (for a final concentration of 1 mM), vortex the sample.
3. Incubate the mixture at 30°C for 15 min with constant agitation.
4. Terminate the reaction by placing the sample on ice and adding 32 µl of 1 M $MgCl_2$ (for a final concentration of 60 mM), vortex the sample.

D Affinity Precipitation of Activated G protein

1. Insert a spin cup into a collection tube for each sample.
2. Swirl the bottle of glutathione resin to thoroughly resuspend the agarose beads. Add 100 µl of the 50% resin slurry to the spin cup with collection tube. Centrifuge the tubes at 6,000xg for 10-30 sec.
3. Discard the flow-through. Add 400 µl of 1X Lysis/Binding/Wash Buffer to each spin cup with resin. Invert the tubes gently several times. Centrifuge the tubes at 6,000xg for 10-30 sec. Discard the flow-through.
4. Thaw the GST-Raf1-RBD on ice. If precipitates are present, centrifuge the tube at 6,000xg for 10-30 sec. Draw off the supernatant and immediately make 80 µg aliquots. Store aliquots for later use at -80°C. Note: Due to the nature of this protein the presence of precipitation is expected and does not impact the performance.
5. Add 80 µg of GST-Raf1-RBD to the spin cup containing the glutathione resin.
6. Immediately transfer up to 700 µl of the cell lysate (containing at least 500 µg of total protein) to the spin cup, close the cap and vortex the sample.
7. Seal cap of the collection tube with laboratory film to prevent leakage, which may result from the presence of detergent in the lysate, vortex the sample.
8. Incubate the reaction mixture at 4°C for 1 hr with gentle rocking.
9. Centrifuge the spin cup with collection tube at 6,000xg for 10-30 sec.
10. Remove the laboratory film and transfer the spin cup to a new collection tube.
11. To wash resin, add 400 µl of 1X Cell Lysis/Binding/Wash Buffer, invert the tube three times, and centrifuge at 6,000xg for 10-30 sec. Discard the buffer. Repeat this wash step two additional times.
12. Transfer the spin cup to a new collection tube.
13. Prepare 50 µl of reducing sample buffer for each pull-down reaction by adding dithiothreitol (DTT) to 2X SDS Sample Buffer to a final concentration of 200 mM.
14. Add 50 µl 2X reducing sample buffer to the resin. Vortex the sample and incubate at room temperature for 2 min.
15. Centrifuge the tube at 6,000xg for 2 min. Remove and discard the spin cup containing the resin.
16. Heat the eluted samples for 5 min at 95-100°C. Samples may be electrophoresed on a gel or stored at -20°C for future use.

E Western blot analysis

NOTE: Volumes are for 10 cm x 10 cm (100 cm^2) of membrane; for different sized membranes, adjust volumes accordingly.

1. Load 20-25 µl onto SDS-PAGE gel (10 cm x 10 cm).
   NOTE: CST recommends loading prestained molecular weight markers (#74124, 10 µl/lane) to verify electrotransfer and biotinylated protein ladder (#7727, 10 µl/lane) to determine molecular weights. 4-20% acrylamide or Tris-glycine gel provides best separation.
2. Electrotransfer to nitrocellulose membrane.
3. After transfer, wash nitrocellulose membrane with 25 ml TBS for 5 min at room temperature.
4. Incubate membrane in 25 ml of blocking buffer (TBS containing 0.1% Tween-20 and 5% BSA) for 1 hr at room temperature.
5. Wash three times for 5 min each with 15 ml of 1X TBST.
6. Incubate membrane with Ras Mouse mAb (1:200 dilution) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
7. Wash three times for 5 min each with 15 ml of 1X TBST.
8. Incubate membrane with Anti-mouse IgG, HRP-linked antibody (1:2000, #7076) and HRP-conjugated anti-biotin antibody (1:1000, #7075) to detect biotinylated protein markers in 10 ml of 1X TBST containing 5% milk with gentle agitation for 1 hour at room temperature.
9. Wash three times for 5 min each with 15 ml of 1X TBST.
10. Incubate membrane with 10 ml LumiGLO® (0.5 ml 20X LumiGLO®, 0.5 ml 20X Peroxide, #7003, and 9.0 ml ddH_2O water) with gentle agitation for 1 min at room temperature. **NOTE:** LumiGLO® substrate can be further diluted if signal response is too fast.
11. Drain membrane of excess developing solution (do not let dry), wrap in plastic wrap and expose to light film. An initial 10-sec exposure should indicate the proper exposure time. **NOTE:** Due to the kinetics of the detection reaction, signal is most intense immediately following LumiGLO® incubation and declines over the following 2 hr.