Rho-GTPase Antibody Sampler Kit

1 Kit (6 x 20 µl)

See www.cellsignal.com for individual component applications, species cross-reactivity, dilutions and additional application protocols.

Description: The Rho-GTPase Antibody Sampler Kit contains reagents to examine aspects of cell migration, adhesion, proliferation and differentiation in cells. The kit includes enough antibody to perform two western blot experiments with each primary antibody.

Background: The Rho family of small GTPases, including Rho, Rac and cdc42, act as molecular switches to regulate processes such as cell migration, adhesion, proliferation and differentiation (1). RhoA, RhoB and RhoC are all highly homologous but appear to have divergent biological functions. The best characterized of these proteins, RhoA, regulates actomyosin contractility, cytokinesis, focal adhesion assembly and cell polarity (2-5). Mammalian Rac exists as three isoforms (Rac1, Rac2 and Rac3) that show high sequence similarity. Rac1 and Rac2 are ubiquitously expressed and play key signaling roles in cytoskeletal reorganization, membrane trafficking, transcriptional regulation and cell growth and development (6). Phosphorylation of Rac1 at a putative Akt site (Ser71) may limit Rac1 activity through inhibition of GTP binding (7), Rac2 is expressed in colts of hematopoietic origin, while Rac3 is highly expressed in brain and in many other tissues. The Vav family of guanine-nucleotide exchange factors mediates activation of Rho/Rac family small GTPases (8). Negative regulation of Rho-activity members of the p190 RhoGAP family (p190-A and p190-B) may be controlled by Src phosphorylation of Tyr residues, activating the p190 GAP domain (8-10).

Furthermore, Rho GDP dissociation inhibitor (RhoGDI) associates with Rho/Rac to negatively regulate nucleotide exchange membrane localization (11).

Specificity/Sensitivity: Cdc42 (11A11) Rabbit mAb detects endogenous levels of total Cdc42 protein and does not cross-react with other small GTPases.

Phospho-Rac1/cdc42 (Ser71) Antibody detects endogenous Rac1/cdc42 only when phosphorylated at Ser71 and may also recognize phospho-RhoA (Ser73).

Rac1/2/3 Antibody detects endogenous levels of total Rac1/2/3 proteins.

RhoA (6789) Rabbit mAb recognizes endogenous levels of total RhoA protein.

RhoB Antibody recognizes endogenous levels of RhoB.

RhoC (D40E4) Rabbit mAb recognizes endogenous levels of RhoC.

Anti-rabbit IgG, HRP-linked Antibody

See www.cellsignal.com for validation data and a complete listing of recommended companion products.

Background References:

U.S. Patent No. 5,675,063

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Western Immunoblotting Protocol

For western blots, incubate membrane with diluted primary antibody in either 5% w/v BSA or nonfat dry milk, 1X TBS, 0.1% Tween® 20 at 4°C with gentle shaking, overnight.

NOTE: Please refer to primary antibody datasheet or product webpage for recommended primary antibody dilution buffer and recommended antibody dilution.

A. Solutions and Reagents

NOTE: Prepare solutions with reverse osmosis deionized (RODI) or equivalent grade water.

1. 20X Phosphate Buffered Saline (PBS): (#9808) To prepare 1 L 1X PBS: add 50 ml 20X PBS to 950 ml dH2O, mix.
2. 10X Tris Buffered Saline (TBS): (#12498) To prepare 1 L 1X TBS: add 100 ml 10X to 900 ml dH2O, mix.
3. 1X SDS Sample Buffer: Prepar blueprint Pack (#7722) or Red Loading Pack (#7723). Prepare fresh 3X reducing loading buffer by adding 1/10 volume 3X DTT to 1 volume of 3X SDS loading buffer. Dilute to 1X with dH2O.
4. 10X Tris-Glycine SDS Running Buffer: (#4050) To prepare 1 L 1X running buffer: add 100 ml 1X running buffer to 900 ml dH2O, mix.
5. 10X Tris-Glycine Transfer Buffer: (#12539) To prepare 1 L 1X transfer buffer: add 100 ml 10X transfer buffer to 200 ml methanol + 700 ml dH2O, mix.
6. 10X Tris Buffered Saline with Tween® 20 (TBST): (#9997) To prepare 1 L 1X TBST: add 100 ml 10X TBST to 900 ml dH2O, mix.
7. Nonfat Dry Milk: (#9999)
8. Blocking Buffer: 1X TBST with 5% w/v nonfat dry milk; for 150 ml, add 7.5 g nonfat dry milk to 150 ml 1X TBST and mix well.
9. Wash Buffer: (#9997) 1X TBST
10. Bovine Serum Albumin (BSA): (#9998)
11. Primary Antibody Dilution Buffer: 1X TBST with 5% BSA or 5% nonfat dry milk as indicated on primary antibody datasheet; for 20 ml, add 1.0 g BSA or nonfat dry milk to 20 ml 1X TBST and mix well.
13. Prestained Protein Marker, Broad Range (Premixed Format): (#7720)
15. Secondary Antibody Conjugated to HRP: anti-rabbit (#7074); anti-mouse (#7076)
16. Detection Reagent: LumiGLO® chemiluminescent reagent and peroxide (#7003) or SignalFire™ ECL Reagent (#6883)

B. Protein Blotting

A general protocol for sample preparation.

1. Treat cells by adding fresh media containing regulator for desired time.
2. Aspirate media from cultures; wash cells with 1X PBS; aspirate.
3. Lyse cells by adding 1X SDS sample buffer (100 µl per well of 6-well plate or 500 µl for a 10 cm diameter plate). Immediately scrape the cells off the plate and transfer the extract to a microcentrifuge tube. Keep on ice.
4. Sonicate for 10–15 sec to complete cell lysis and shear DNA (to reduce sample viscosity).
5. Heat a 20 µl sample to 95–100°C for 5 min; cool on ice.
6. Microcentrifuge for 5 min.
7. Load 20 µl onto SDS-PAGE gel (10 cm x 10 cm). NOTE: Loading of prestained molecular weight markers (#7720, 10 µl/tane) to verify electrophoresis and biotinylated protein ladder (#7727, 10 µl/tane) to determine molecular weights are recommended.
8. Electrophoresis to nitrocellulose membrane (#12369).

C. Membrane Blocking and Antibody Incubations

NOTE: Volumes are for 10 cm x 10 cm (100 cm²) of membrane; for different sized membranes, adjust volumes accordingly.

I. Membrane Blocking

1. (Optional) After transfer, wash nitrocellulose membrane with 25 ml TBS for 5 min at room temperature.
2. Incubate membrane in 25 ml of blocking buffer for 1 hr at room temperature.
3. Wash three times for 5 min each with 15 ml of TBST.

II. Primary Antibody Incubation

1. Incubate membrane and primary antibody (at the appropriate dilution and diluent as recommended in the product datasheet) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
2. Wash three times for 5 min each with 15 ml of TBST.
3. Incubate membrane with the species appropriate HRP-conjugated secondary antibody (#7074 or #7076 at 1:2000) and anti-biotin, HRP-linked Antibody (#7075 at 1:1000–1:3000) to detect biotinylated protein markers in 10 ml of blocking buffer with gentle agitation for 1 hr at room temperature.
4. Wash three times for 5 min each with 15 ml of TBST.
5. Proceed with detection (Section D).

D. Detection of Proteins

1. Incubate membrane with 10 ml LumiGLO® 0.5 ml 20X LumiGLO® #7003, 0.5 ml 20X peroxide, and 9.0 ml purified water) or 10 ml SignalFire™ #6883 (5 ml Reagent A, 5 ml Reagent B) with gentle agitation for 1 min at room temperature.
2. Drain membrane of excess developing solution (do not let dry), wrap in plastic wrap and expose to x-ray 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 incubation and declines over the following 2 hr.