Product Manual

RhoA Activation Assay Kit

Catalog Number

STA-403-A	20 assays

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

CELL BIOLABS, INC.
Creating Solutions for Life Science Research

Introduction

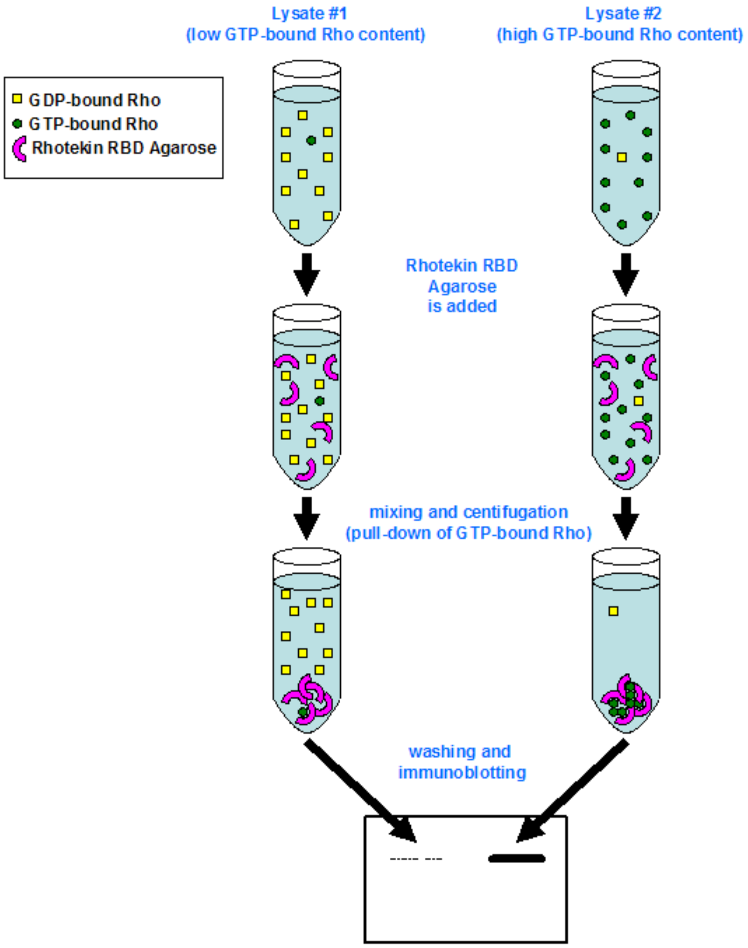
Small GTP-binding proteins (or GTPases) are a family of proteins that serve as molecular regulators in signaling transduction pathways. RhoA, a 21 kDa protein, regulating a variety of biological response pathways that include cell growth, cell transformation and tumor invasion. Like other small GTPases, RhoA regulates molecular events by cycling between an inactive GDP-bound form and an active GTP-bound form. In its active (GTP-bound) state, RhoA binds specifically to the Rho-binding domain (RBD) of Rhotekin to control downstream signaling cascades.

Cell Biolabs' RhoA Activation Assay Kit utilizes Rhotekin RBD Agarose beads to selectively isolate and pull-down the active form of Rho from purified samples or endogenous lysates. Subsequently, the precipitated GTP-Rho is detected by western blot analysis using an anti-RhoA specific monoclonal antibody (see Figure 3 and Assay Principle).

Cell Biolabs' RhoA Activation Assay Kit provides a simple and fast tool to monitor the activation of RhoA. The kit includes easily identifiable Rhotekin RBD Agarose beads (see Figure 1), pink in color, and a RhoA Immunoblot Positive Control for quick RhoA identification. Each kit provides sufficient quantities to perform 20 assays.

Figure 1:Rhotekin RBD Agarose beads, in color, are easy to visualize, minimizing potential loss during washes and aspirations.

Assay Principle



Related Products

1. STA-400: Pan-Ras Activation Assay Kit
2. STA-401-1: Rac1 Activation Assay
3. STA-401-2: Rac2 Activation Assay
4. STA-405: RhoA/Rac1/Cdc42 Activation Assay Combo Kit
5. STA-410: Raf1 RBD Agarose Beads

Kit Components

1. Rhotekin RBD Agarose (Part No. STA-412): One vial – 800 µL of 50% slurry, 400 µg Rhotekin RBD in PBS containing 50% glycerol.

   Note: Agarose bead appears pink in color for easy identification, washing, and aspiration.

2. 100X GTPγS (Part No. 240103): One vial – 50 µL of 10 mM GTPγS dissolved in sterile water.

3. 100X GDP (Part No. 240104): One vial – 50 µL of 100 mM GDP dissolved in sterile water.

4. 5X Assay/Lysis Buffer (Part No. 240102): One bottle – 30 mL of 125 mM HEPES, pH 7.5, 750 mM NaCl, 5% Igepal CA-630, 50 mM $MgCl_2$, 5 mM EDTA, 10% Glycerol.

5. Anti-RhoA, Mouse Monoclonal (Part No. 240302): One vial – 40 µL in PBS, pH 7.4, 0.05% NaN_3, 0.1% BSA.

   Note: This monoclonal antibody specifically reacts with human, mouse, and rat RhoA.

6. RhoA Immunoblot Positive Control (Part No. 240310): One vial – 100 µL of partially purified, recombinant RhoA from *E. coli* (provided ready-to-use in 1X reducing SDS-PAGE Sample Buffer, pre-boiled).

Materials Not Supplied

1. Stimulated and non-stimulated cell lysates
2. RhoA activators
3. Protease inhibitors
4. 0.5 M EDTA in water
5. 1 M $MgCl_2$
6. 30°C incubator or water bath
7. 4°C tube rocker or shaker
8. 2X reducing SDS-PAGE sample buffer
9. Electrophoresis and immunoblotting systems
10. Immunoblotting wash buffer such as TBST (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.05% Tween-20)
11. Immunoblotting blocking buffer (TBST containing 5% Non-fat Dry Milk)
12. PVDF or nitrocellulose membrane
13. Secondary Antibody
14. ECL Detection Reagents

Storage

Store all kit components at -20ºC. The 5X Assay/Lysis Buffer may be stored at either -20ºC or 4ºC. Avoid multiple freeze/thaw cycles.

Preparation of Reagents

- 1X Assay/Lysis Buffer: Mix the 5X Stock briefly and dilute to 1X in deionized water. Just prior to usage, add protease inhibitors such as 1 mM PMSF, 10 µg/mL leupeptin, and 10 µg/mL aprotinin.

Preparation of Samples

Note: It is advisable to use fresh cell lysates because GTP-RhoA is quickly hydrolyzed to GDP-RhoA; frozen lysates stored at -70°C may be used. Performing steps at 4°C or on ice may reduce hydrolysis. Avoid multiple freeze/thaw cycles of lysates.

I. Adherent Cells

1. Culture cells to approximately 80-90% confluence. Stimulate cells with RhoA activator(s) as desired.
2. Aspirate the culture media and wash twice with ice-cold PBS.
3. Completely remove the final PBS wash and add ice-cold 1X Assay/Lysis Buffer to the cells (0.5 - 1 mL per 100 mm tissue culture plate).
4. Place the culture plates on ice for 10-20 minutes.
5. Detach the cells from the plates by scraping with a cell scraper.
6. Transfer the lysates to appropriate size tubes and place on ice.
7. If nuclear lysis occurs, the cell lysates may become very viscous and difficult to pipette. If this occurs, lysates can be passed through a 27½-gauge syringe needle 3-4 times to shear the genomic DNA.
8. Clear the lysates by centrifugation for 10 minutes (14,000 x g at 4°C).
9. Collect the supernatant and store samples on ice for immediate use, or snap freeze and store at -70°C for future use.
10. Proceed to GTPγS/GDP Loading for positive and negative controls, or Pull-Down Assay.

II. Suspension Cells

1. Culture cells and stimulate with RhoA activator(s) as desired.
2. Perform a cell count, and then pellet the cells by centrifugation.
3. Aspirate the culture media and wash twice with ice-cold PBS.
4. Completely remove the final PBS wash and add ice-cold 1X Assay/Lysis Buffer to the cell pellet (0.5 – 1 mL per 1 x 10^7 cells).
5. Lyse the cells by repeated pipetting.
6. Transfer the lysates to appropriate size tubes and place on ice.
7. If nuclear lysis occurs, the cell lysates may become very viscous and difficult to pipette. If this occurs, lysates can be passed through a 27½-gauge syringe needle 3-4 times to shear the genomic DNA.
8. Clear the lysates by centrifugation for 10 minutes (14,000 x g at 4°C).

9. Collect the supernatant and store samples on ice for immediate use, or snap freeze and store at -70°C for future use.
10. Proceed to GTPγS/GDP Loading for positive and negative controls, or Pull-Down Assay.

Assay Protocol

Important Note: Before running any Small GTPase pulldown assay, it is always a good practice to run a Western Blot directly on the cell lysate using the antibody provided in this kit. For example: load 5 µg, 10 µg and 20 µg of lysate onto an SDS-PAGE gel, transfer and blot. When proceeding with the pulldown assay, use 100-times the amount of lysate that gave you a clear band of your desired small GTPase in the direct Western blot. For example: if the 5 µg band was faint but the 10 µg band was clear and strong, use 100 x 10 µg = 1 mg of lysate in the assay. Using sufficient lysate in the pulldown assay is critical to success.

I. GTPγS/GDP Loading (Positive and Negative Controls)

Note: Samples that will not be GTPγS/GDP loaded may be kept on ice during the loading of controls.

1. Aliquot 0.5 – 1 mL of each cell lysate to two microcentrifuge tubes.

   Note: Typical protein content/sample is > 0.5 mg.
2. Adjust the volume of each sample to 1 mL with 1X Assay Lysis Buffer.
3. Add 20 µL of 0.5 M EDTA to each sample.
4. Add 10 µL of 100X GTPγS to one tube (positive control) and 10 µL of 100X GDP to the other tube (negative control). Mix and label each tube appropriately.
5. Incubate the tubes for 30 minutes at 30°C with agitation.
6. Stop the loading by adding 65 µL of 1 M $MgCl_2$ to each tube. Mix and place tubes on ice.
7. Continue with Pull-Down assay.

II. RhoA Pull-Down Assay

1. Aliquot 0.5 – 1 mL of cell lysate (treated with RhoA activators or untreated) to a microcentrifuge tube.
2. Adjust the volume of each sample to 1 mL with 1X Assay Lysis Buffer.
3. Thoroughly resuspend the Rhotekin RBD Agarose bead slurry by vortexing or titurating.
4. Quickly add 40 µL of resuspended bead slurry to each tube (including GTPγS/GDP controls).
5. Incubate the tubes at 4°C for 1 hour with gentle agitation.
6. Pellet the beads by centrifugation for 10 seconds at 14,000 x g.
7. Aspirate and discard the supernatant, making sure not to disturb/remove the bead pellet.
8. Wash the bead 3 times with 0.5 mL of 1X Assay Buffer, centrifuging and aspirating each time.
9. After the last wash, pellet the beads and carefully remove all the supernatant.
10. Resuspend the bead pellet in 40 µL of 2X reducing SDS-PAGE sample buffer.
11. Boil each sample for 5 minutes.

12. Centrifuge each sample for 10 seconds at 14,000 x g.

III. Electrophoresis and Transfer

1. Load 20 µL/well of pull-down supernatant to a polyacrylamide gel. Also, it's recommended to include a pre-stained MW standard (as an indicator of a successful transfer in step 3).

   Note: If desired, 10 µL/well of RhoA Immunoblot Positive Control (provided ready-to-use, pre-boiled) can be added as an immunoblot positive control.

2. Perform SDS-PAGE as per the manufacturer's instructions.
3. Transfer the gel proteins to a PVDF or nitrocellulose membrane as per the manufacturer's instructions.

IV. Immunoblotting and Detection (all steps are at room temperature, with agitation)

1. Following the electroblotting step, immerse the PVDF membrane in 100% Methanol for 15 seconds, and then allow it to dry at room temperature for 5 minutes.

   Note: If Nitrocellulose is used instead of PVDF, this step should be skipped.

2. Block the membrane with 5% non-fat dry milk in TBST for 1 hr at room temperature with constant agitation.

   Incubate the membrane with Anti-RhoA Antibody, freshly diluted 1:200 to 1:1000 in 5% non-fat dry milk/TBST, for 1-2 hr at room temperature with constant agitation.

   Note: To conserve antibody, incubations should be performed in a plastic bag.

3. Wash the blotted membrane three times with TBST, 5 minutes each time.
4. Incubate the membrane with a secondary antibody (e.g. Goat Anti-Mouse IgG, HRP-conjugate), freshly diluted in 5% non-fat dry milk/TBST, for 1 hr at room temperature with constant agitation.
5. Wash the blotted membrane three times with TBST, 5 minutes each time.
6. Use the detection method of your choice. We recommend enhanced chemiluminescence reagents from Pierce.

Example of Results

The following figure demonstrates typical results seen with Cell Biolabs RhoA Activation Assay Kit. One should use the data below for reference only.

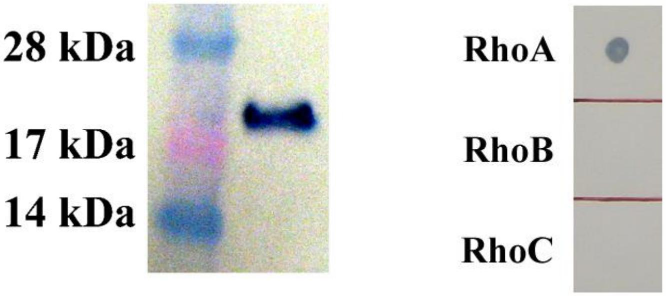


Figure 2: RhoA Activation Assay. *Left Image*: RhoA Immunoblot Positive Control. *Right Image*: Demonstrates Anti-RhoA monoclonal antibody specificity by dot blot.

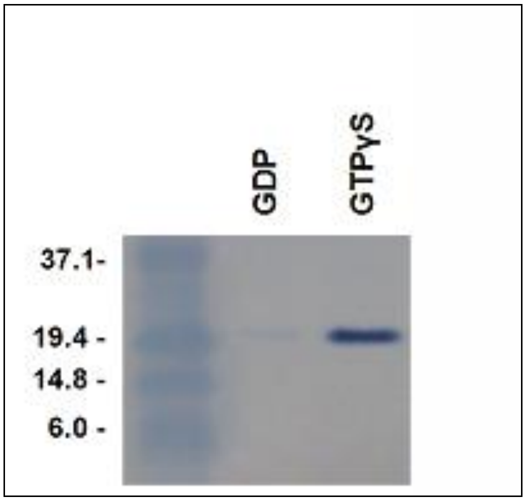


Figure 3: RhoA Activation Assay. *Lane 1*: MW Standard. *Lane 2*: MDA-231 cell lysate loaded with GDP and incubated with Rhotekin RBD Agarose beads. *Lane 3*: MDA-231 cell lysate loaded with GTPγS and incubated with Rhotekin RBD Agarose beads.

Reference

1. Ren X.D. and Schwartz M. A. (2000) *Methods Enzymol*. **325**, 264-72.

Recent Product Citations

1. Kim, K.B. et al. (2022). WNT5A-RHOA signaling is a driver of tumorigenesis and represents a therapeutically actionable vulnerability in small cell lung cancer. *Cancer Res*. doi: 10.1158/0008-5472.CAN-22-1170.
2. Engelmann, J. et al. (2022). Regulation of bone homeostasis by MERTK and TYRO3. *Nat Commun*. **13**(1):7689. doi: 10.1038/s41467-022-33938-x.
3. Chen, K.J. et al. (2022). Somatic A-to-I RNA-edited RHOA isoform 2 specific-R176G mutation promotes tumor progression in lung adenocarcinoma. *Mol Carcinog*. doi: 10.1002/mc.23490.

4. Khan, A. et al. (2021). Tumor necrosis factor-induced ArhGEF10 selectively activates RhoB contributing to human microvascular endothelial cell tight junction disruption. *FASEB J*. **35**(6): e21627. doi: 10.1096/fj.202002783RR.
5. Pan, W. et al. (2021). The Regulatory Subunit PPP2R2A of PP2A Enhances Th1 and Th17 Differentiation through Activation of the GEF-H1/RhoA/ROCK Signaling Pathway. *J Immunol*. doi: 10.4049/jimmunol.2001266.
6. Mossa, A.H. et al. (2021). Deleterious impact of nerve growth factor precursor (proNGF) on bladder urothelial and smooth muscle cells. *Cell Signal*. doi: 10.1016/j.cellsig.2021.109936.
7. Kim, J.H. et al. (2021). Gamma subunit of complement component 8 is a neuroinflammation inhibitor. *Brain*. doi: 10.1093/brain/awaa425.
8. Xu, J. et al. (2021). Acute glucose influx-induced mitochondrial hyperpolarization inactivates myosin phosphatase as a novel mechanism of vascular smooth muscle contraction. *Cell Death Dis*. **12**(2):176. doi: 10.1038/s41419-021-03462-9.
9. Wang, F. et al. (2020). Topical administration of rapamycin promotes retinal ganglion cell survival and reduces intraocular pressure in a rat glaucoma model. *Eur J Pharmacol*. doi: 10.1016/j.ejphar.2020.173369.
10. Isaksen, T.J. et al. (2020). Repulsive Guidance Molecule A Suppresses Adult Neurogenesis. *Stem Cell Reports*. pii: S2213-6711(20)30093-X. doi: 10.1016/j.stemcr.2020.03.003.
11. Buonpane, C. et al. (2020). ROCK1 Inhibitor Stabilizes E-cadherin and Improves Barrier Function in Experimental Necrotizing Enterocolitis. *Am J Physiol Gastrointest Liver Physiol*. doi: 10.1152/ajpgi.00195.2019.
12. Phung, B. et al. (2019). The X-Linked DDX3X RNA Helicase Dictates Translation Reprogramming and Metastasis in Melanoma. *Cell Rep*. **27**(12):3573-3586.e7. doi: 10.1016/j.celrep.2019.05.069.
13. Yoon, S. et al. (2019). EPHB6 mutation induces cell adhesion-mediated paclitaxel resistance via EPHA2 and CDH11 expression. *Exp Mol Med*. **51**(6):61. doi: 10.1038/s12276-019-0261-z.
14. Gao, S. et al. (2019). Histidine-rich glycoprotein ameliorates endothelial barrier dysfunction through regulation of NF-κB and MAPK signal pathway. *Br J Pharmacol*. doi: 10.1111/bph.14711.
15. Kang, M. et al. (2019). Roles of CD133 in microvesicle formation and oncoprotein trafficking in colon cancer. *FASEB J*. **33**(3):4248-4260. doi: 10.1096/fj.201802018R.
16. Soliman, M. et al. (2018). Rotavirus-Induced Early Activation of the RhoA/ROCK/MLC Signaling Pathway Mediates the Disruption of Tight Junctions in Polarized MDCK Cells. *Sci Rep*. **8**(1):13931. doi: 10.1038/s41598-018-32352-y.
17. Sorrentino, S. et al. (2018). Hindlimb Ischemia Impairs Endothelial Recovery and Increases Neointimal Proliferation in the Carotid Artery. *Sci Rep*. **8**(1):761. doi: 10.1038/s41598-017-19136-6.
18. Huang, X. et al. (2018). RhoA-stimulated intra-capillary morphology switch facilitates the arrest of individual circulating tumor cells. *Int J Cancer*. **142**(10):2094-2105. doi: 10.1002/ijc.31238.
19. Ye, Y. et al. (2016). Down-regulation of 14-3-3 Zeta Inhibits TGF-β1-Induced Actomyosin Contraction in Human Trabecular Meshwork Cells Through RhoA Signaling Pathway. *Invest Ophthalmol Vis Sci*. **57**(2):719-30. doi: 10.1167/iovs.15-17438.
20. Ma, J. H. et al. (2016). The role of IRE-XBP1 pathway in regulation of retinal pigment epithelium tight junctionsXBP1 regulates the RPE tight junctions. *Invest Ophthalmol Vis Sci*. **57**:5244-5252.
21. Kim, J. M. et al. (2016). Distinctive and selective route of PI3K/PKCα-PKCδ/RhoA-Rac1 signaling in osteoclastic cell migration. *Mol Cell Endocrinol*. doi: 10.1016/j.mce.2016.08.042.

22. Cui, J. et al. (2016). Epigenetic silencing of TPM2 contributes to colorectal cancer progression upon RhoA activation. *Tumor Biol*. doi:10.1007/s13277-016-5103-1.
23. Ye, Y. et al. (2016). Down-regulation of 14-3-3 Zeta inhibits TGF-β1–induced actomyosin contraction in human trabecular meshwork cells through rhoa signaling pathway14-3-3 zeta regulates actomyosin contraction in TM cells. *Invest Ophthalmol Vis Sci.* **57**:719-730.
24. Gayle, S. et al. (2015). piggyBac insertional mutagenesis screen identifies a role for nuclear RhoA in human ES cell differentiation. *Stem cell Reports.* doi: 10.1016/j.stemcr.2015.03.001.
25. Choi, D. S. et al. (2015). SDF-1α stiffens myeloma bone marrow mesenchymal stromal cells through the activation of RhoA-ROCK-Myosin II. *Int J Cancer.* **136**: E219-E229.
26. Schmidt, L. & Carrillo-Sepulveda, M. A. (2015). Toll-like receptor 2 mediates vascular contraction and activates RhoA signaling in vascular smooth muscle cells from STZ-induced type 1 diabetic rats. *Pflügers Arch.* doi: 10.1007/s00424-015-1688-2.
27. Fujita, M. et al. (2014). Nitric oxide increases the invasion of pancreatic cancer cells via activation of the PI3K–AKT and RhoA pathways after carbon ion irradiation. *FEBS Lett.* **588**:3240-3250.
28. Tripathi, V. et al. (2014). DLC1 suppresses NF-κB activity in prostate cancer cells due to its stabilizing effect on adherens junctions. *Springerplus.* **3**:27.
29. Kwak, S. Y. et al. (2014). Ionizing radiation-inducible miR-494 promotes glioma cell invasion through EGFR stabilization by targeting p190B RhoGAP. *Biochim Biophys Acta.* **1843**:508-516.
30. Cao, X. et al. (2014). Resveratrol prevents AngII-induced hypertension via AMPK activation and RhoA/ROCK suppression in mice. *Hypertens Res*. **37**:803-810.

Warranty

These products are warranted to perform as described in their labeling and in Cell Biolabs literature when used in accordance with their instructions. THERE ARE NO WARRANTIES THAT EXTEND BEYOND THIS EXPRESSED WARRANTY AND CELL BIOLABS DISCLAIMS ANY IMPLIED WARRANTY OF MERCHANTABILITY OR WARRANTY OF FITNESS FOR PARTICULAR PURPOSE. CELL BIOLABS' sole obligation and purchaser's exclusive remedy for breach of this warranty shall be, at the option of CELL BIOLABS, to repair or replace the products. In no event shall CELL BIOLABS be liable for any proximate, incidental or consequential damages in connection with the products.

Contact Information

Cell Biolabs, Inc.
5628 Copley Drive
San Diego, CA 92111
Worldwide: +1 858 271-6500
USA Toll-Free: 1-888-CBL-0505
E-mail: tech@cellbiolabs.com
www.cellbiolabs.com