—A helping hand for your research

Configuration-specific Monoclonal Antibody Based

RhoA Activation Assay Kit

(30 Assays)

Cat. # 80601

Address: NewEast Biosciences
24 Whitewoods Lane
Malvern, PA 19355

Orders: 610-945-2007
sale@neweastbio.com
Support: 610-945-2007
info@neweastbio.com
Web: www.neweastbio.com

RhoA Activation Assay Kit Protcol

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FOR RESEARCH USE ONLY NOT FOR USE IN DIAGNOSTIC PROCEDURES

Product Description

Small GTPases are a super-family of cellular signaling regulators. RhoA belongs to the Rho sub-family of GTPases that regulate cell motility, cell division, and gene transcription. GTP binding increases the activity of RhoA, and the hydrolysis of GTP to GDP renders it inactive.

Currently the activation of RhoA proteins is assayed with the binding of GTP-bound RhoA to the p21-binding domain (PBD) of p21-activated protein kinase (PAK). This method is based on the observation that the active, GTP-bound RhoA could bind to the PBD of PAK. However, the reproducibility of this method is poor. This is partially due to the relatively quick hydrolysis of GTP to GDP during the assay procedure, and the low binding affinity of PBD to RhoA-GTP.

NewEast Biosciences RhoA Activation Assay Kit is based on the configuration-specific monoclonal antibody that specifically recognizes RhoA-GTP, but not RhoA-GDP. Given the high affinity of monoclonal antibodies to their antigens, the activation assay could be performed in a much shorter time. This assay provides the reliable results with consistent reproducibility.

These anti-RhoA-GTP monoclonal antibody can also be used to monitor the activation of RhoA in cells and in tissues by immunohistochemistry.

NewEast Biosciences RhoA Activation Assay Kit provides a simple and fast method to monitor the activation of RhoA. Each kit provides sufficient quantities to perform 30 assays.

Assay Principle

NewEast Biosciences RhoA Activation Assay Kit bases on the configuration-specific anti-RhoA-GTP monoclonal antibody to measure the active RhoA-GTP levels, either from cell extRhoAts or from in vitro GTPγS loading RhoA activation assays. Briefly, anti-active RhoA mouse monoclonal antibody will be incubated with cell lysates containing RhoA-GTP. The bound active RhoA will then be pulled down by protein A/G agarose. The precipitated active RhoA will be detected by immunoblot analysis using anti-RhoA rabbit polyclonal antibody.

Kit Contents

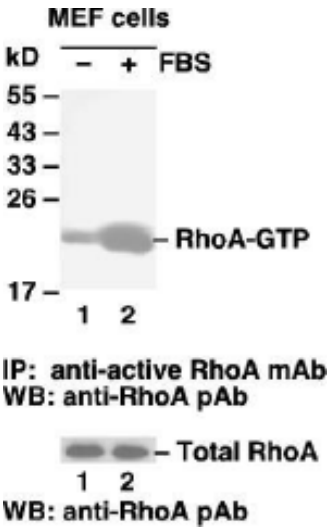
This kit contains enough reagents for approximately 30–35 pull–down assays.

Reagent	Cat. #	Quantity	Storage
Anti – active RhoA Mouse Monoclonal Antibody	Cat. # 26904	1 X 35µl	–20℃
Protein A/G Agarose	Cat. # 30301	1X600µl	4℃
5X Assay/Lysis Buffer	Cat. # 30303	1X30mL	4℃
Anti– RhoA Rabbit polyclonal Antibody	Cat.# 21017	1X50µl	–20℃
100x GTPγS	Cat. # 30302	1X50µl	–80℃
100x GDP	Cat. # 30304	1X50µl	–80℃
HRP– Goat Anti–Rabbit IgG	Cat. # 29002	1X50µl	–20℃

Note: For GDP and GTPrS, aliquot into 10x5ul volumes, then store at–80 degrees.

Example of Results

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



RhoA activation assay. MEF cells were treated with (lane 2) or without (lane 1) PDGF. Cell lysates were incubated with an anti-active RhoA monoclonal antibody (Cat. # 26904) (top panel). The precipitated active RhoA was immunoblotted with an anti-RhoA rabbit polyclonal antibody (Cat # 21017). The bottom panel shows the Western blot with anti-RhoA of the cell lysates used (5% of that used in the top panel).

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Materials Needed but Not Supplied

Stimulated and non–stimulated cell lysates
Protease inhibitors
4℃ tube rocker or shaker
0.5 M EDTA, pH8.0
1 M MgCl2
2X reducing SDS–PAGE sample buffer
Electrophoresis and immunoblotting systems
Immunoblotting wash buffer such as TBST
(10 mM Tris–HCl, pH 7.4, 0.15 M NaCl, 0.05% Tween–20)
Immunoblotting blocking buffer
(TBST containing 5% Non–fat Dry Milk or 3% BSA)
ECL Detection Reagents

A Reagent Preparation

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

B Sample Preparation

Adherent Cells

1. Culture cells (one 10–cm plate, ~ 107 cells) to approximately 80–90% confluence. Stimulate cells with activator or inhibitor 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 10 cm 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 (12,000 x g at 4℃).
9. Collect the supernatant and store samples (~1–2 mg of total proteins) on ice for immediate use, or snap freeze and store at – 70℃ for future use.

Suspension Cells

1. Culture cells and stimulate with activator or inhibitor 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 107cells).
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
8. Clear the lysates by centrifugation for 10 minutes (12,000 xg at 4℃).
9. Collect the supernatant and store samples on ice for immediate use, or snap freeze and store at – 70℃ for future use.

C In vitro GTPγS/GDP Protein Loading for positive and negative controls

Note: In vivo stimulation of cells will activate approximately 10% of the available Rhoa, whereas in vitro GTPγS protein loading will activate nearly 90% of Rhoa.

1. Aliquot 0.5 ml of each cell extract to two microfuge tubes (or use 1μg of purified Rhoa protein).
2. To each tube, add 20 μl of 0.5 M EDTA (to 20 mM final concentration).
3. Add 5 μl of 100 X GTPγS (to 100 μM, final concentration) to one tube (positive control).
4. Add 5 μl of 100 X GDP (to 1 mM, final concentration) to the second tube (negative control).
5. Incubate the tubes at 30℃ for 30 minutes with agitation.
6. Stop loading by placing the tubes on ice and adding 32.5 μl of 1 M MgCl2 (to 60 mM, final concentration).

D Affinity Precipitation of Activated G protein

1. Aliquot 0.5 – 1 mL of cell lysate (~1 mg of total cellular protein) to a microcentrifuge tube.
2. Adjust the volume of each sample to 1 mL with 1X Assay/Lysis Buffer.
3. Add 1μl anti–active Rhoa monoclonal antibody to the tube.
4. Thoroughly resuspend the protein A/G Agarose bead slurry by vortexing or titurating.
5. Quickly add 20 μL of resuspended bead slurry to each tube.
6. Incubate the tubes at 4℃ for 1 hour with gentle agitation.
7. Pellet the beads by centrifugation for 1 min at 5,000 x g .
8. Aspirate and discard the supernatant, making sure not to disturb/remove the bead pellet.
9. Wash the bead 3 times with 0.5 mL of 1X Assay/Lysis Buffer, centrifuging and aspirating each time.
10. After the last wash, pellet the beads and carefully remove all the supernatant.
11. Resuspend the bead pellet in 20 μL of 2X reducing SDS–PAGE sample buffer.
12. Boil each sample for 5 minutes.
13. Centrifuge each sample for 10 seconds at 5,000 x g

E Western blot analysis

1. Load 15 μL/well of pull–down supernatant to a polyacrylamide gel (17%). Also, it's recommended to include a pre–stained MW standard (as an indicator of a successful transfer in step 3).
2. Perform SDS–PAGE following the manufacturer's instructions.
3. Transfer the gel proteins to a PVDF or nitrocellulose membrane following the manufacturer's instructions
4. 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.
5. Block the membrane with 5% non–fat dry milk or 3% BSA in TBST for 1 hr at room temperature with constant agitation. Incubate the membrane with anti– Rhoa polyclonal antibody, freshly diluted 1:50~500 (depending on the amount of Rhoa proteins in your samples) in 5% non–fat dry milk or 3% BSA/TBST, for1–2 hr at room temperature with constant agitation or at 4℃ overnight.
6. Wash the blotted membrane three times with TBST, 5 minutes each time.
7. In cubate the membrane with a secondary antibody (e.g. Goat Anti–Rabbit IgG, HRP–conjugate), freshly diluted 1:1000 in 5% non–fat dry milk or 3% BSA/TBST, for 1 hr at room temperature with constant agitation.
8. Wash the blotted membrane three times with TBST, 5 minutes each time.
9. Use the detection method of your choice such as ECL