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**Configuration-specific Monoclonal Antibody Based**

**RhoA Activation Assay Kit**

**(30 Assays)**

**Cat. # 80601**

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**FOR RESEARCH USE ONLY. NOT FOR DIAGNOSTIC APPLICATIONS**

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# RhoA Activation Assay Kit Protocol

## Cat. # 80601

## Introduction

### A. Background

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.

### B. Assay Principle

NewEast Biosciences RhoA Activation Assay Kit uses configuration-specific anti-RhoA-GTP Mouse monoclonal antibody to measure RhoA-GTP levels either from cell extracts or from in vitro GTPγS loading RhoA activation assays. Anti-RhoA-GTP mouse monoclonal antibody is first incubated with cell lysates containing RhoA-GTP. Next, the GTP-bound RhoA is pulled down by protein A/G agarose. Finally, the precipitated RhoA-GTP is detected through immunoblot analysis using Anti-RhoA Rabbit Polyclonal Antibody.

### C. Kit Contents

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

Reagent	Cat. #	Quantity	Storage
Anti-RhoA-GTP Mouse Monoclonal Antibody	26904	30 µL	-20°C
Protein A/G Agarose	30301	600 µL	4°C
5X Assay/Lysis Buffer	30302	30 mL	4°C
Anti-RhoA Rabbit Polyclonal Antibody	21017	50 µL	-20°C
100X GTPγS	30303	50 µL	-20°C
100X GDP	30304	50 µL	-20°C
HRP-Goat Anti-Rabbit IgG	29002	50 µL	-20°C

### D. Materials Needed but Not Supplied

1. Stimulated and non-stimulated cell lysates
2. Protease inhibitors
3. 4°C tube rocker or shaker
4. 0.5 M EDTA at pH 8.0
5. 1.0 M MgCl<sub>2</sub>
6. 2X reducing SDS-PAGE sample buffer
7. Electrophoresis and immunoblotting systems
8. Immunoblotting wash buffer such as TBST (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.05% Tween-20)
9. Immunoblotting blocking buffer (TBST containing 5% Non-fat Dry Milk or 3% BSA)
10. ECL Detection Reagents

### E. Example Results

The following figure demonstrates example results seen with the RhoA Activation Assay Kit. 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-RhoA-GTP monoclonal antibody (Cat. # 26904) (top panel). The precipitated RhoA-GTP 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).

## Assay Procedure

### A. Reagent Preparation

**1X Assay/Lysis Buffer:** Shake the 5X Stock Buffer (Cat. # 30302) briefly and dilute with 4 times deionized water to make 1X buffer. Just prior to usage, add protease inhibitors such as 1 mM PMSF, 10 µg/mL leupeptin, and 10 µg/mL aprotinin.

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### B. Sample Preparation

#### Adherent Cells

1. Culture cells (one 10-cm plate) to approximately 80-90% confluence ( $\sim 10^7$  cells). Stimulate the cells with activator or inhibitor as desired.
2. Aspirate the culture media and wash twice with ice-cold PBS buffer.
3. Completely remove the final PBS wash and add ice-cold 1X Assay/Lysis Buffer (See Reagent Preparation) to 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 them on ice.
7. If nuclear lysis occurs, the cell lysates may become 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 centrifuging at 12,000 x g and 4°C for 10 minutes.
9. Collect the supernatant into a tube and store the sample ( $\sim 1$ -2 mg of total protein) on ice for immediate use, or snap freeze and store it at -70°C for the 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 through 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 (See Reagent Preparation) to the cell pellet (0.5-1 mL per  $10^7$  cells).
5. Lyse the cells by repeated pipetting.
6. Transfer the lysates to appropriate size tubes and place them on ice.
7. If nuclear lysis occurs, the cell lysates may become 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 centrifuging at 12,000 x g and 4°C for 10 minutes.  
Collect the supernatant into a tube and store the sample ( $\sim 1$ -2 mg of total protein) on ice for immediate use, or snap freeze and store it at -70°C for the future use

### C. In vitro GTPγS/GDP Protein 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 cell extract (or 1 μg of purified RhoA protein) into two microcentrifuge tubes.
2. To each tube, add 20 μL of 0.5 M EDTA (final concentration of 20 mM).
3. Add 5 μL of 100 X GTPγS (Cat. # 30303) to the first tube as a positive control.
4. Add 5 μL of 100 X GDP (Cat. # 30304) to the second tube as a negative control.
5. Incubate both tubes at 30°C for 30 minutes with agitation.
6. Stop loading by placing the tubes on ice and adding 32.5 μL of 1 M MgCl<sub>2</sub> (final concentration of 60 mM).

### D. Affinity Precipitation of Activated G Protein

1. Aliquot 0.5-1 mL of cell lysates (about 1 mg of total cellular protein) to a microcentrifuge tube.
2. Adjust the volume to 1 mL with 1X Assay/Lysis Buffer (See Reagent Preparation).
3. Add 1 μL anti-RhoA-GTP antibody (Cat. # 26904).
4. Prepare the protein A/G Agarose bead slurry (Cat. # 30301) by resuspending through vortexing or titrating.
5. Quickly add 20 μL of resuspended bead slurry to above tube.
6. Incubate the tube at 4°C for 1 hour with gentle agitation.
7. Pellet the beads through centrifugation at 5,000 x g for 1 min.
8. Aspirate and discard the supernatant (making sure not to disturb or remove the bead pellet).
9. Wash the beads 3 times with 0.5 mL of 1X Assay/Lysis Buffer, centrifuging and aspirating each time.
10. After the third wash, pellet the beads through centrifugation and carefully remove all the supernatant.
11. Resuspend the bead pellet in 20 μL of 2X reducing SDS-PAGE sample buffer.
12. Boil the sample for 5 minutes.
13. Centrifuge it at 5,000 x g for 10 seconds.

### E. Western Blot Analysis

1. Load 15 μL/well of pull-down supernatant to a polyacrylamide gel (17%). It is recommended to include Prestained Protein Markers (as an indicator of a successful transfer in step 3 below).
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.  
**Note:** Steps 4-11 are at room temperature with agitation
4. Following electroblotting, 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, step 4 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.
6. Wash the blotted membrane three times with TBST, 5 minutes each time.
7. Incubate the membrane with Anti-RhoA Rabbit Polyclonal Antibody (Cat. # 21017), which is freshly diluted 1:50~500 (depending on the amount of RhoA proteins in your sample) in 5% non-fat dry milk or 3% BSA in TBST, for 1-2 hr at room temperature with constant agitation or at 4°C overnight.
8. Wash the blotted membrane three times with TBST, 5 minutes each time.
9. Incubate the membrane with a secondary antibody (Cat. # 29002), which is freshly diluted 1:1000 in 5% non-fat dry milk or 3% BSA in TBST, for 1 hr at room temperature with constant agitation.
10. Wash the blotted membrane three times with TBST, 5 minutes each time.
11. Use the detection method of your choice such as ECL.