



Pioneering GTPase and Oncogene Product Development since 2010

**Configuration-specific Monoclonal Antibody Based
Ras Activation Assay Kit
(30 Assays)
Cat. # 81101**

FOR RESEARCH USE ONLY. NOT FOR DIAGNOSTIC APPLICATIONS

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Ras Activation Assay Kit Protocol

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Introduction

A. Background

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

Currently the activation of Ras proteins is assayed with the binding of GTP-bound Ras to the Ras-binding domain (RBD) of Raf protein kinase. This method is based on the observation that the active, GTP-bound Ras could bind to the RBD of Raf. 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 RBD to Ras-GTP.

NewEast Biosciences Ras Activation Assay Kit is based on the configuration-specific monoclonal antibody that specifically recognizes Ras-GTP, but not Ras-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.

B. Assay Principle

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

C. Kit Contents

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

Reagent	Cat. #	Quantity	Storage
Anti-Ras-GTP Mouse Monoclonal Antibody	26909	30 μL	-20°C
Protein A/G Agarose	30301	600 μL	4°C
5X Assay/Lysis Buffer	30302	30 mL	4°C
Anti-Ras Rabbit Polyclonal Antibody	21021	50 μL	-20°C
100X GTPγS	30303	50 μL	-20°C
100X GDP	30304	50 μL	-20°C

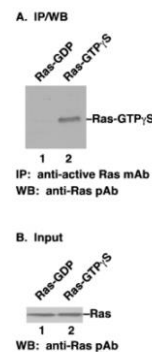
HRP-Goat Anti-Rabbit IgG	29002	50 μL	-20°C
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D. Materials Needed but Not Supplied

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 MgCl₂
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 Ras Activation Assay Kit. For reference only.



Ras Activation Assay. Purified Ras protein was loaded with GDP (lane 1) or GTPγS (lane 2). These proteins were incubated with an anti-Ras-GTP monoclonal antibody (Cat. # 26905) (top panel). The precipitated active Ras was immunoblotted with an anti-Ras rabbit polyclonal antibody (Cat # 21021). The bottom panel shows the Western blot with anti-Ras of the used Ras-GDP and Ras-GTPγS.

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

μL of 1 M MgCl_2 (final concentration of 60 mM).

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 27 $\frac{1}{2}$ -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 (~ 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 27 $\frac{1}{2}$ -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 (~ 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 GTPyS/GDP Protein for Positive and Negative controls

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

1. Aliquot 0.5 mL of cell extract (or 1 μg of purified Ras 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 GTPyS (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

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-Ras-GTP antibody (Cat. # 26909).
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-Ras Rabbit Polyclonal Antibody (Cat. # 21021), which is freshly diluted 1:50~500 (depending on the amount of Ras 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.