



Pioneering GTPase and Oncogene Product Development since 2010

**Configuration-specific Monoclonal Antibody Based
RafA Activation Assay Kit
(30 Assays)
Cat. # 83601**

FOR RESEARCH USE ONLY. NOT FOR DIAGNOSTIC APPLICATIONS

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RalA Activation Assay Kit Protocol

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Introduction

A. Background

Small GTPases are a super-family of cellular signaling regulators. The Ras-like small G proteins, RalA/B, are important components of Ras signaling pathways, implicated in the initiation and maintenance of tumorigenic transformation, as well as vesicle transport, apoptosis, transcription, cell migration, and cell proliferation.

Currently there is no direct assay to measure the activation of Ral GTPases.

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

B. Assay Principle

NewEast Biosciences RalA Activation Assay Kit uses configuration-specific anti-Ral-GTP Mouse monoclonal antibody to measure Ral-GTP levels either from cell extracts or from in vitro GTP γ S loading Ral activation assays. Anti-Ral-GTP mouse monoclonal antibody is first incubated with cell lysates containing Ral-GTP. Next, the GTP-bound Ral is pulled down by protein A/G agarose. Finally, the precipitated active RalA or RalB will be detected by immunoblot analysis using anti-RalA or RalB rabbit polyclonal antibody, respectively.

C. Kit Contents

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

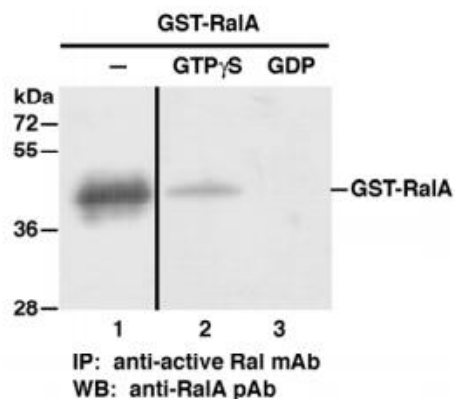
Reagent	Cat. #	Quantity	Storage
Anti-Ral-GTP Mouse Monoclonal Antibody	26913	30 μ L	-20°C
Protein A/G Agarose	30301	600 μ L	4°C
5X Assay/Lysis Buffer	30302	30 mL	4°C
Anti-RalA Rabbit Polyclonal Antibody	21034	50 μ L	-20°C
100X GTP γ S	30303	50 μ L	-20°C
100X GDP	30304	50 μ L	-20°C
HRP-Goat Anti-Rabbit IgG	29002	50 μ L	-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 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 RalA Activation Assay Kit. For reference only.



RalA Activation Assay. Purified GST-RalA proteins were immunoprecipitated after treated with GDP (lane 3) or GTP γ S (lane 2). Lane 1 was GST-RalA protein loaded as control. Immunoprecipitation was done with the anti-Ral-GTP mouse monoclonal antibody (Cat. No. 26913). Immunoblot was with an anti-RalA rabbit polyclonal antibody (Cat. No. 21034).

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 μ g/mL leupeptin, and 10 μ g/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 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 GTP γ S/GDP Protein for Positive and Negative controls

Note: In vivo stimulation of cells will activate approximately 10% of the available Ral, whereas in vitro GTP γ S protein loading will activate nearly 90% of Ral.

1. Aliquot 0.5 mL of cell extract (or 1 μ g of purified RalA protein) into two microcentrifuge tubes.
2. To each tube, add 20 μ L of 0.5 M EDTA (final concentration of 20 mM).
3. Add 5 μ L of 100 X GTP γ S (Cat. # 30303) to the first tube as a positive control.
4. Add 5 μ L 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 μ L of 1 M MgCl $_2$ (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 μ L anti-Ral-GTP antibody (Cat. # 26913).
4. Prepare the protein A/G Agarose bead slurry (Cat. # 30301) by resuspending through vortexing or titrating.
5. Quickly add 20 μ L 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 μ L 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 μ L/well of pull-down supernatant to a polyacrylamide gel (17%). It is recommended to include Prestained Protein Marker (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-RalA Rabbit Polyclonal Antibody (Cat. # 21034), which is freshly diluted 1:50~500 (depending on the amount of RalA 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.