—A helping hand for your research

Configuration-specific Monoclonal Antibody Based

RalA Activation Assay Kit

(30 Assays)

Cat. # 83601

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

RalA Activation Assay Kit Protcol

Cat# 83601

FOR RESEARCH USE ONLY NOT FOR USE IN DIAGNOSTIC PROCEDURES

Product Description

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.

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

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

Assay Principle

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

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

Kit Contents

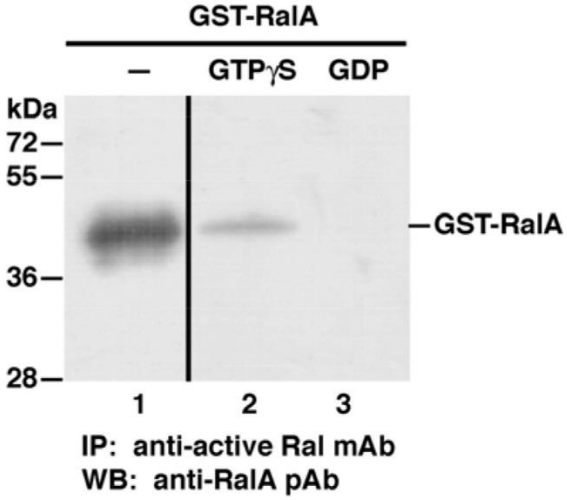
This kit contains enough reagents for approximately 30–35 pull–down assays.

Reagent	Cat. #	Quantity	Storage
Anti – active RalA Mouse Monoclonal Antibody	Cat. # 26913	1 X 35µl	–20℃
Protein A/G Agarose	Cat. # 30301	1X600µl	4℃
5X Assay/Lysis Buffer	Cat. # 30303	1X30mL	4℃
Anti– RalA Rabbit polyclonal Antibody	Cat.# 21034	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 RalA Activation Assay Kit. One should use the data below for reference only.



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

RalA Activation Assay Kit Protcol

Cat# 83601

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 GTP γ S/GDP Protein Loading for positive and negative controls

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

1. Aliquot 0.5 ml of each cell extract to two microfuge tubes (or use 1μg of purified RalA 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 GTP γ S (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 Ral 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– RalA polyclonal antibody, freshly diluted 1:50~500 (depending on the amount of RalA 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