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Product Manual

Configuration-specific Monoclonal Antibody Based

Rap1 Activation Assay Kit

Catalog Number：81401

20 assays

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Product Description

Small GTPases are a super-family of cellular signaling regulators. The small Ras-like GTPase Rap1 is an evolutionary conserved protein that originally gained interest because of its capacity to revert the morphological phenotype of Ras-transformed fibroblasts. Rap1 is regulated by a large number of stimuli that include growth factors and cytokines, but also physical force and osmotic stress. Rap1 was shown to regulate multiple basic cellular processes. The best studied aspect of Rap1 function in endothelial cells involved its role in regulation of cell-cell junction formation and remodeling.

Currently there is no direct assay to measure the activation of Rap1 GTPases.

NewEast Biosciences Rap1 Activation Assay Kit is based on the configuration-specific monoclonal antibody that specifically recognizes Rap1-GTP, but not Rap1-GDP. 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-Rap1-GTP monoclonal antibodies can also be used to monitor the activation of Rap1 in cells and in tissues by immunohistochemistry.

NewEast Biosciences Rap1 Activation Assay Kit provides a simple and fast method to monitor the activation of Rap1. Each kit provides sufficient quantities to perform 20 assays.

Assay Principle

NewEast Biosciences Rap1 Activation Assay Kit bases on the configuration-specific anti-Rap1-GTP monoclonal antibody to measure the active Rap1-GTP levels, either from cell extracts or from in vitro GTPγS loading Rap1 activation assays. Briefly, anti-active Rap1 mouse monoclonal antibody will be incubated with cell lysates containing Rap1-GTP. The bound active Rap1 will then be pulled down by protein A/G agarose. The precipitated active Rap1 will be detected by immunoblot analysis using anti-Rap1 rabbit polyclonal antibody.

Kit Components

1. Anti-active Rap1, Mouse Monoclonal Antibody (Catalog No. 26912): One vial – 22 µL (1 mg/ml) in PBS, pH 7.4, containing 50% glycerol and 0.05% sodium azide. This antibody

specifically recognizes Rap1-GTP from all vertebrates.

2. Protein A/G Agarose (Catalog No. 30301): One vial – 400 µL of 50% slurry.

3. 5X Assay/Lysis Buffer (Catalog No. 30302): One bottle – 30 mL of 250 mM Tris-HCl, pH 8, 750 mM NaCl, 50 mM $MgCl_2$, 5 mM EDTA, 5% Triton X-100.

4. Anti-Rap1, Rabbit Polyclonal Antibody (Catalog No. 21104): One vial – 100 µL (1 mg/ml) in PBS, pH 7.4, contained 50% glycerol.

5. 100 X GTPγS (Catalog No. 30303): One vial –100 µl at 10 mM, use 5 µL of GTPγS for GTP-labeling of 0.5 mL of cell lysate.

6. 100 X GDP (Catalog No. 30304): One vial –100 µl at 100 mM, use 5 µL of GDP for GDP-labeling of 0.5 mL of cell lysate.

Storage

Store all kit components at 4°C until their expiration dates.

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, pH8.0
5. 1 M $MgCl_2$
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. PVDF or nitrocellulose membrane
11. Secondary Antibody
12. ECL Detection Reagents

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.

Sample Preparation

Adherent Cells

1. Culture cells (one 10-cm plate, ~ 10^7 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 °C).
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 °C 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 10^7cells).
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 genomic DNA.
8. Clear the lysates by centrifugation for 10 minutes (12,000 x g at 4 °C).
9. Collect the supernatant and store samples on ice for immediate use, or snap freeze and store at -70 °C for future use.

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

Note: In vivo stimulation of cells will activate approximately 10% of the available Rap1, whereas in vitro GTPγS protein loading will activate nearly 90% of the Rap1.

1, Aliquot 0.5 ml of each cell extract to two microfuge tubes (or use 1 µg of purified Rap1 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°C for 30 minutes with agitation.

6, Stop loading by placing the tubes on ice and adding 32.5 µl of 1 M $MgCl_2$ (to 60 mM, final concentration).

Assay Procedure

I. Active Rap1 Pull-Down Assay

1. Aliquot 0.5 – 1 mL of cell lysate to a microcentrifuge tube.
2. Adjust the volume of each sample to 1 mL with 1X Assay/Lysis Buffer.
3. Add 1 µl anti-active Rap1 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 °C 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.

II. Electrophoresis and Transfer

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.

III. Immunoblotting and Detection (all steps are at room temperature, with agitation)

1. 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.

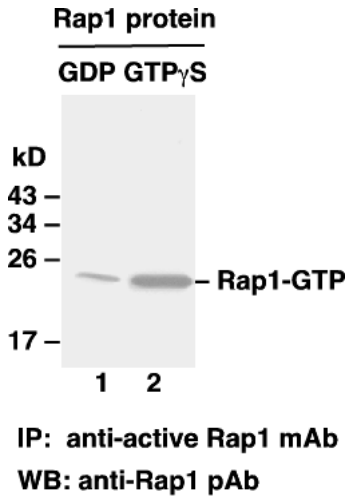
   Note: If Nitrocellulose is used instead of PVDF, this step should be skipped.

2. 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-Rap1 polyclonal antibody, freshly diluted 1:50~1000 (depending on the amount of Rap1 proteins in your samples) in 5% non-fat dry milk or 3% BSA/TBST, for 1-2 hr at room temperature with constant agitation or at 4^{o}C overnight.
3. Wash the blotted membrane three times with TBST, 5 minutes each time.
4. Incubate 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.
5. Wash the blotted membrane three times with TBST, 5 minutes each time.
6. Use the detection method of your choice such as ECL.

Example of Results

The following figure demonstrates typical results seen with NewEast Biosciences Rap1 Activation Assay Kit. One should use the data below for reference only.



Rap1 activation assay. Purified Rap1 proteins were immunoprecipitated after treated with GDP (lane 1) or GTPγS (lane 2). Immunoprecipitation was done with the anti-active Rap1 monoclonal antibody (Cat. No. 26912). Immunoblot was with an anti-Rap1 polyclonal antibody (Cat. No. 21104).