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Product Manual

Configuration-specific Monoclonal Antibody Based

Rab5A Activation Assay Kit

Catalog Number：81301

20 assays

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Product Description

Small GTPases are a super-family of cellular signaling regulators. Rab5 is a member of the Rab-family GTPases. Rab5 is a rate-limiting component of the machinery regulating the kinetics of membrane traffic in the early endocytic pathway.

Currently there is no direct assay to measure the activation of Rab5A GTPases.

NewEast Biosciences Rab5A Activation Assay Kit is based on the configuration-specific monoclonal antibody that specifically recognizes Rab5-GTP, but not Rab5-GDP, and a Rab5A 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-Rab5-GTP monoclonal antibodies can also be used to monitor the activation of Rab5 in cells and in tissues by immunohistochemistry.

NewEast Biosciences Rab5A Activation Assay Kit provides a simple and fast method to monitor the activation of Rab5A. Each kit provides sufficient quantities to perform 20 assays.

Assay Principle

NewEast Biosciences Rab5A Activation Assay Kit bases on the configuration-specific anti-Rab5-GTP monoclonal antibody to measure the active Rab5-GTP levels, either from cell extracts or from in vitro GTPγS loading Rab5 activation assays. Briefly, anti-active Rab5 mouse monoclonal antibody will be incubated with cell lysates containing Rab5-GTP. The bound active Rab5 will then be pulled down by protein A/G agarose. The precipitated active Rab5A will be detected by immunoblot analysis using anti-Rab5A specific rabbit polyclonal antibody.

Kit Components

1. Anti-active Rab5, Mouse Monoclonal Antibody (Catalog No. 26911): One vial – 22 µL (1 mg/ml) in PBS, pH 7.4, containing 50% glycerol and 0.05% sodium azide. This antibody specifically recognizes Rab5-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. 30303): 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-Rab5A, Rabbit Polyclonal Antibody (Catalog No. 21036): One vial – 100 µL (0.2 mg/ml) in PBS, pH 7.4, contained 50% glycerol.

5. 100 X GTPγS (Catalog No. 30302): 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 Rab5, whereas in vitro GTPγS protein loading will activate nearly 90% of the Rab5.

1, Aliquot 0.5 ml of each cell extract to two microfuge tubes (or use 1 μg of purified full length Rab5A 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 Rab5 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 Rab5 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.

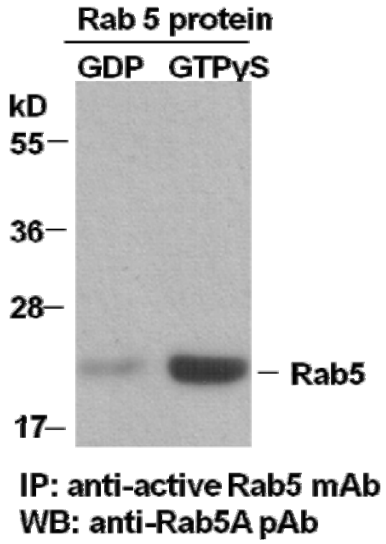
   Note: If Nitrocellulose is used instead of PVDF, this step should be skipped.

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-Rab5A polyclonal antibody, freshly diluted 1:50~1000 (depending on the amount of Rab5A 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°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 Rab5A Activation Assay Kit. One should use the data below for reference only.



Rab5A activation assay. Purified full length Rab5A proteins were immunoprecipitated after treated with GDP (lane left) or GTPγS (lane right). Immunoprecipitation was done with the anti-active

Rab5 monoclonal antibody (Cat. No. 26911). Immunoblot was with an anti-Rab5A polyclonal antibody(Cat. No. 21036).