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Configuration-specific Monoclonal Antibody Based

## **Rab5 Activation Assay Kit**

(30 Assays)

**Cat. # 83701**

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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

# Rab5 Activation Assay Kit Protocol

Cat# 83701

FOR RESEARCH USE ONLY NOT FOR USE IN DIAGNOSTIC PROCEDURES

## 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 Rab5 GTPases.

NewEast Biosciences Rab5 Activation Assay Kit is based on the configuration-specific monoclonal antibody that specifically recognizes Rab5-GTP, but not Rab5-GDP, and a Rab5 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 Rab5 Activation Assay Kit provides a simple and fast method to monitor the activation of Rab5. Each kit provides sufficient quantities to perform 30 assays.

## Assay Principle

NewEast Biosciences Rab5 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 GTP $\gamma$ S 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 Rab5 will be detected by immunoblot analysis using anti-Rab5 specific rabbit polyclonal antibody.

## Kit Contents

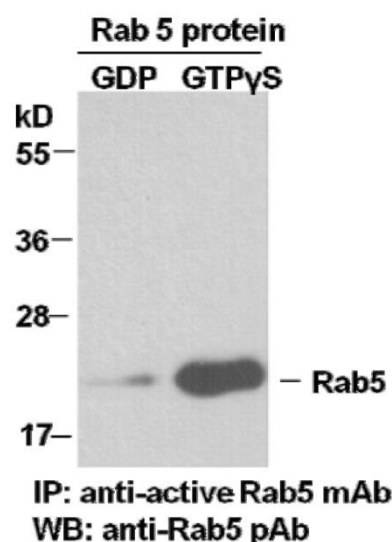
This kit contains enough reagents for approximately 30–35 pull-down assays.

| Reagent                                      | Cat. #       | Quantity       | Storage |
|--|--------------|----------------|---------|
| Anti – active Rab5 Mouse Monoclonal Antibody | Cat. # 26911 | 1 X 35 $\mu$ l | -20°C   |
| Protein A/G Agarose                          | Cat. # 30301 | 1X600 $\mu$ l  | 4°C     |
| 5X Assay/Lysis Buffer                        | Cat. # 30303 | 1X30mL         | 4°C     |
| Anti– Rab5 Rabbit polyclonal Antibody        | Cat. # 21151 | 1X50 $\mu$ l   | -20°C   |
| 100x GTP $\gamma$ S                          | Cat. # 30302 | 1X50 $\mu$ l   | -80°C   |
| 100x GDP                                     | Cat. # 30304 | 1X50 $\mu$ l   | -80°C   |
| HRP– Goat Anti–Rabbit IgG                    | Cat. # 29002 | 1X50 $\mu$ l   | -20°C   |

Note: For GDP and GTP $\gamma$ S, aliquot into 10x5ul volumes, then store at -80 degrees.

## Example of Results

The following figure demonstrates typical results seen with NewEast Biosciences Rab5 Activation Assay Kit. One should use the data below for reference only.



**Rab5 activation assay.** Purified full-length Rab5 proteins were immunoprecipitated after treated with GDP (lane left) or GTP $\gamma$ S (lane right). Immunoprecipitation was done with the anti-active Rab5 monoclonal antibody (Cat. No. 26911). Immunoblot was with an anti-Rab5 polyclonal antibody (Cat. No. 21151).

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## Materials Needed but Not Supplied

Stimulated and non-stimulated cell lysates  
Protease inhibitors  
4°C tube rocker or shaker  
0.5 M EDTA, pH8.0  
1 M MgCl<sub>2</sub>  
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°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 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°C).
9. Collect the supernatant and store samples on ice for immediate use, or snap freeze and store at – 70°C for future use.

## C In vitro GTP $\gamma$ S/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 GTP  $\gamma$  S protein loading will activate nearly 90% of Rab5.

1. Aliquot 0.5 ml of each cell extract to two microfuge tubes (or use 1 µg of purified Rab5 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  $\gamma$  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°C for 30 minutes with agitation.
6. Stop loading by placing the tubes on ice and adding 32.5 µl of 1 M MgCl<sub>2</sub> (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 1 µl anti-active Rab5 monoclonal antibody to the tube.
4. Thoroughly resuspend the protein A/G Agarose bead slurry by vortexing or titrating.
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

## 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-Rab5 polyclonal antibody, freshly diluted 1:50–500 (depending on the amount of Rab5 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.
6. Wash the blotted membrane three times with TBST, 5 minutes each time.
7. 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.
8. Wash the blotted membrane three times with TBST, 5 minutes each time.
9. Use the detection method of your choice such as ECL