Pioneering GTPase and Oncogene Product Development since 2010

Configuration-specific Monoclonal Antibody Based

Rac Activation Assay Kit

(30 Assays)

Cat. # 80501

FOR RESEARCH USE ONLY. NOT FOR DIAGNOSTIC APPLICATIONS

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Rac Activation Assay Kit Protocol
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Introduction

A. Background

Small GTPases are a super-family of cellular signaling regulators. Rac belongs to the Rho sub-family of GTPases that regulate cell motility, cell division, and gene transcription. GTP binding increases the activity of Rac, and the hydrolysis of GTP to GDP renders it inactive.

Currently the activation of Rac proteins is assayed with the binding of GTP-bound Rac to the p21-binding domain (PBD) of p21-activated protein kinase (PAK). This method is based on the observation that the active, GTP-bound Rac could bind to the PBD of PAK. However, the reproducibility of this method is poor. This is partially due to the relatively quick hydrolysis of GTP to GDP during the assay procedure, and the low binding affinity of PBD to Rac-GTP.

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

B. Assay Principle

NewEast Biosciences Rac Activation Assay Kit uses configuration-specific anti-Rac-GTP Mouse monoclonal antibody to measure Rac-GTP levels either from cell extracts or from in vitro GTPγS loading Rac activation assays. Anti-Rac-GTP mouse monoclonal antibody is first incubated with cell lysates containing Rac-GTP. Next, the GTP-bound Rac is pulled down by protein A/G agarose. Finally, the precipitated Rac-GTP is detected through immunoblot analysis using Anti-Rac Rabbit Polyclonal Antibody.

C. Kit Contents

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

Reagent	Cat. #	Quantity	Storage
Anti-Rac-GTP Mouse Monoclonal Antibody	26903	30 µL	-20°C
Protein A/G Agarose	30301	600 µL	4°C
5X Assay/Lysis Buffer	30302	30 mL	4°C
Anti-Rac Rabbit Polyclonal Antibody	21003	50 µL	-20°C
100X GTPγS	30303	50 µL	-80°C
100X GDP	30304	50 µL	-80°C
HRP-Goat Anti-Rabbit IgG	29002	50 µL	-20°C

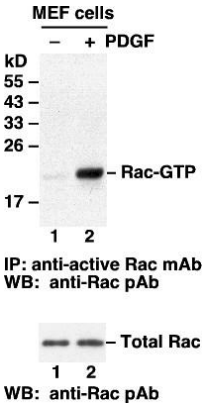
For best results, aliquot GTPγS and GDP at first use to minimize freeze/thaw cycles.

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 $MgCl_2$
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. ECL Detection Reagents

E. Example Results

The following figure demonstrates example results seen with the Rac Activation Assay Kit. For reference only.



Rac Activation Assay. MEF cells were treated with (lane 2) or without (lane 1) PDGF. Cell lysates were incubated with an anti-Rac-GTP monoclonal antibody (Cat. # 26903) (top panel). The precipitated active Rac was immunoblotted with an anti-Rac rabbit polyclonal antibody (Cat # 21003). The bottom panel shows the Western blot with anti-Rac of the cell lysates used (5% of that used in the top panel).

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 µg/mL leupeptin, and 10 µg/mL aprotinin.

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B. Sample Preparation

Adherent Cells

1. Culture cells (one 10-cm plate) to approximately 80-90% confluence (~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 27½-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 27½-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 GTPγS/GDP Protein for Positive and Negative controls (Optional)

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

1. Aliquot 0.5 mL of cell extract (or 1 µg of purified Rac protein) into two microcentrifuge tubes.
2. To each tube, add 20 µL of 0.5 M EDTA (final concentration of 20 mM).
3. Add 5 µL of 100 X GTPγS (Cat. # 30303) to the first tube as a positive control.
4. Add 5 µL 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 µL 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 µL anti-Rac-GTP antibody (Cat. # 26903).
4. Prepare the protein A/G Agarose bead slurry (Cat. # 30301) by resuspending through vertexing or titrating.
5. Quickly add 20 µL of resuspended bead slurry to above tube.
6. Incubate the tube at 4°C for 1 hr 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 µL 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 µL/well of pull-down supernatant to a polyacrylamide gel (17%). It is recommended to include Prestained Protein Markers (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-Rac Rabbit Polyclonal Antibody (Cat. # 21003), which is freshly diluted 1:50~500 (depending on the amount of Rac 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.