ab173245
Rap1 Activation Assay Kit

Instructions for Use

For the simple and fast measurement of Rap1 activation.

This product is for research use only and is not intended for diagnostic use.

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1. Background

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.
2. Principle of the Assay

Abcam’s Ras 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. 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.

ab173245 uses an anti-Rap1-GTP monoclonal antibody to measure the active Rap1-GTP levels, either from cell extracts or from in vitro GTP gamma S loading Rap1 activation assays. Briefly, the 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 and the precipitated active Rap1 will be detected by immunoblot analysis using anti-Rap1 rabbit polyclonal antibody.
3. Protocol Summary

Preparation:
- Prepare buffers
- Prepare samples and positive/negative controls

Active Rap1 Pull-Down Assay:
1. Add sample to microcentrifuge tube
2. Add anti-active Rap1 Mouse monoclonal antibody
3. Add re-suspended protein A/G Agarose bead slurry
4. Incubate tubes at 4°C for 1 hour (with gentle agitation)
5. Pellet beads by centrifugation
6. Wash pellet 3 times with 1X Assay/Lysis Buffer
7. Pellet beads by centrifugation
9. Boil and centrifuge each sample
Electrophoresis and Transfer

Load pull-down supernatant to polyacrylamide gel.

Perform SDS-PAGE following your preferred protocol.

Immerse PVDF membrane in 100% Methanol before immersing it in transfer buffer.

Transfer gel proteins to a PVDF or nitrocellulose membrane following your preferred protocol.

Immunoblotting and Detection

Block the membrane with 5% non-fat dry milk or 3% BSA in TBST

Incubate membrane with anti-Rap1 Rabbit polyclonal antibody

Wash membrane three times with TBST

Incubate membrane with the secondary antibody

Wash membrane three times with TBST

Use detection method of your choice (e.g. ECL)
4. Materials Supplied

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-active Rap1, Mouse Monoclonal Antibody</td>
<td>1 x 22 µL</td>
</tr>
<tr>
<td>Note: This antibody specifically recognizes Rap1-GTP from all vertebrates</td>
<td></td>
</tr>
<tr>
<td>Protein A/G Agarose</td>
<td>1 x 400 µL</td>
</tr>
<tr>
<td>5X Assay/Lysis Buffer</td>
<td>2 x 15 mL</td>
</tr>
<tr>
<td>Anti-Rap1, Rabbit polyclonal Antibody</td>
<td>1 x 100 µL</td>
</tr>
<tr>
<td>100X GTP gamma S (10 mM)</td>
<td>1 x 100 µL</td>
</tr>
<tr>
<td>100X GDP (100 mM)</td>
<td>1 x 100 µL</td>
</tr>
</tbody>
</table>

5. Storage

Store all kit components at 4°C.
6. Materials Required, Not Supplied

- Stimulated and non-stimulated cell lysates
- Protease inhibitors – we recommend Protease Inhibitor Cocktail (ab65621)
- 4°C tube rocker or shaker
- PBS, ice-cold
- 0.5 M EDTA, pH 8.0
- 1 M MgCl$_2$
- 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)
- PVDF or nitrocellulose membrane
- Secondary Antibody against Rap1 polyclonal antibody – we recommend Goat polyclonal Secondary Antibody to Rabbit IgG Fc (HRP) (ab98467)
- ECL Detection Reagents – we recommend Optiblot ECL Detect kit (ab133406)
7. Reagent Preparation

1X Assay/Lysis Buffer:
Mix the 5X Stock briefly and dilute to 1X in deionized water and keep cold. Just prior to usage, add protease inhibitors such as 1 mM PMSF, 10 µg/mL leupeptin, and 10 µg/mL aprotinin.

Protein A/G Agarose:
Cut the end of a 200 µl tip and resuspend the bead slurry prior use.

8. Sample Preparation

A. Adherent Cells
1. Culture cells (10⁷ cells, ~ 1x10-cm plate) 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/ 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 -80°C for future use.

B. 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 10^7 cells).

5. Lyse the cells by repeated pipetting up and down.

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 -80°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 Rap1, whereas *in vitro* GTP gamma S protein loading will activate nearly 90% of 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 (20 mM final concentration).

3. Positive control: add 5 μL of 100X GTP gamma S (100 μM, final concentration) to one tube.

4. Negative control: add 5 μL of 100X GDP (to 1 mM, final concentration) to the second tube.

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 MgCl₂ (60 mM, final concentration).
9. Assay Procedure

A. Active Rap1 Pull-Down Assay

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 Rap1 Mouse monoclonal antibody to the tube.
4. Thoroughly resuspend the protein A/G Agarose bead slurry by vortexing or pipetting up and down.
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.
B. Electrophoresis and Transfer

1. Load 15 μL/well of pull-down supernatant to a 17% polyacrylamide gel.
   Note: 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 your preferred protocol.

3. Following the transfer step, immerse the PVDF membrane in 100% Methanol for 15 seconds before immersing it in transfer buffer.
   Note: If Nitrocellulose is used instead of PVDF, this step should be skipped.

4. Transfer the gel proteins to a PVDF or nitrocellulose membrane following your preferred protocol.

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

1. Block the membrane with 5% non-fat dry milk or 3% BSA in TBST for 1 hr at room temperature with constant agitation.

2. Incubate the membrane with anti-Rap1 Rabbit polyclonal antibody, freshly diluted 1/50 – 1/1000 (depending on the amount of Rap1 proteins in your samples) in 5% non-fat dry milk or 3% BSA/TBST, for 1-2 hours at room temperature with constant agitation or at 4°C overnight.

3. Wash the membrane three times with TBST, 5 minutes each time.
4. Incubate the membrane with the secondary antibody freshly
diluted in 5% non-fat dry milk or 3% BSA/TBST for
30 min – 1 hour at room temperature with constant agitation.
Use the dilution appropriate for your secondary antibody (for
example – Goat polyclonal Secondary Antibody to Rabbit IgG
Fc (HRP) (ab98467) can be use at 1/5000 – 1/25000).
5. Wash the membrane three times with TBST, at least
5 minutes each time.
6. Use the detection method of your choice, such as ECL.
10. Data Analysis

Example results for Western blotting

Note: The following figure demonstrates typical results seen with Abcam’s Rap1 Activation Assay Kit. Data below should be used for reference only.

Rap1 Activation Assay. Purified Rap1 proteins were immunoprecipitated after treatment with GDP (lane 1) or GTPγS (lane 2).
Immunoprecipitation: Proteins were incubated with the anti-active Rap1 monoclonal antibody.
Immunoblot: Precipitated active Rap1 was immunoblotted with the anti-Rap1 polyclonal antibody provided in the kit.