ab139586

Rac Activation Kit

Instructions for Use

For the rapid, sensitive and accurate quantification of active Rac in various samples

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

Members of the Rac/Rho subfamily of small GTPases or G-proteins are key regulators of numerous processes related to cytoskeletal dynamics. For example, Rac1/2 and Cdc42 (and other closely related members) regulate cell migration, contraction and cell volume, among other features, through the control of the cortical actin cytoskeleton. Cdc42 is furthermore a major player in the establishment of a polarized phenotype in numerous specialized cell types whereas Rac is a critical regulator of NADPH oxidases and attendant ROS production.

In common with most other small G-proteins, cellular Rac and Cdc42 cycle between an inactive GDPbound and an active GTP-bound state. Cellular Rac-GDP/GTP levels are tightly controlled by guanine nucleotide exchange factors (GEFs), which activate Rac by promoting GTP uptake, and GTP hydrolase activating proteins (GAPs), which accelerate conversion of Rac-bound GTP to GDP. A third group of Rac regulating proteins, the RhoGDIs, controls Rac activity by governing their availability for association with membranes.
Traditionally, GTPase activity measurements involved metabolic labelling of cells with inorganic $[^{32}\text{P}]$-phosphate followed by isolation of the GTPase and chromatographic analysis of bound guanine nucleotides. This methodology provides quantitative data for GDP and GTP levels on Rac but is a tedious and time consuming procedure that requires working with large amounts of radioactivity and is prone to various sources of errors.

More recently an alternative non-radioactive technique has been described that exploits the selective interaction of the Cdc42/Rac interactive binding domain (CRIB) of the Rac-effector PAK1 with the active, Rac-GTP conformation. Recombinant, GST-tagged PAK-CRIB is added to cell extracts to pull out Rac-GTP, which is consequently detected by Western blotting. This approach has greatly accelerated and thus simplified semi-quantitative Rac activity determinations.
Abcam’s Rac Activation Kit provides a rapid, cost-effective and reliable tool for detection and semi-quantitative analysis of the cellular activation state of Rac1 and Rac2 G-proteins. If combined with immunological detection of Cdc42, the kit can also detect activated Cdc42-GTP. Please note that this kit provides only an antibody for the detection of Rac1. This assay is suitable for 10 x 6 well plates.

Active Rac, i.e. Rac complexed to GTP, is purified out of cell extracts via a one-step batch affinity purification step employing GST-PAK-CRIB, an affinity probe that selectively interacts with the active, GTP-bound Rac conformation in loss of the inactive Rac-GDP complex.
The kit further includes recombinant Rac1ΔC-term that serves dual roles:

1. It can be used as western blot control for immunodetection

2. Upon loading with GDP or GTP-γ-S (also included in this kit), Rac1ΔC-GDP or Rac1ΔC-GTP-γ-S complexes can be used to “spike” cell extracts thus serving as internal assay controls of signal specificity.

2. Assay Summary

- Prepare Cell Lysate
- Prepare Rac-GDP and/or Rac-GTP-γ-S Control (Optional)
- Process Lysate for SDS-PAGE
- Perform Western Blot Analysis
### 3. Components and Storage

#### A. Kit Contents

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Storage Temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x Lysis Buffer stock</td>
<td>30 mL</td>
<td>4°C</td>
</tr>
<tr>
<td>100 x Protease inhibitor mix</td>
<td>650 μL</td>
<td>-20°C</td>
</tr>
<tr>
<td>GST-PAK-CRIB*</td>
<td>1.5 mg</td>
<td>-80°C</td>
</tr>
<tr>
<td>GDP, 10 mM in water,</td>
<td>100 μL</td>
<td>-20°C</td>
</tr>
<tr>
<td>GDP, 100 mM in water,</td>
<td>100 μL</td>
<td>-20°C</td>
</tr>
<tr>
<td>GTP-γ-S, 10 mM in water,</td>
<td>100 μL</td>
<td>-20°C</td>
</tr>
<tr>
<td>Ral nucleotide loading solution (NLS)</td>
<td>2 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>1 M MgCl$_2$</td>
<td>100 μL</td>
<td>-20°C</td>
</tr>
<tr>
<td>Rac1ΔC (residues 1-178) in 50% glycerol solution</td>
<td>25 μg</td>
<td>-20°C</td>
</tr>
<tr>
<td>Glutathione-Sepharose slurry</td>
<td>5 mL</td>
<td>4°C</td>
</tr>
<tr>
<td>Rac rabbit polyclonal antibody</td>
<td>50 μL</td>
<td>4°C</td>
</tr>
</tbody>
</table>
*Upon arrival, aliquot GST-PAK-CRIB and store at -80°C to avoid repeated freeze/thaw cycles.

B. Additional Materials Required

- Secondary anti-mouse IgG antibody:
  
  *We recommend Rabbit polyclonal Secondary Antibody to Mouse IgG-H&amp;L (HRP) (ab97046) or Goat polyclonal Secondary Antibody to Mouse IgG-H&amp;L (HRP) (ab97023)*

- Western signal detection system

- Standard reagents/instruments for SDS-PAGE and Western Blot analysis.
  
  *We recommend our Optiblot range of products:*

  - Optiblot SDS Gel 4-20% (10 x 10cm) (ab119205)
  - Optiblot SDS Gel 4-20% (8 x 10cm) (ab119209)
  - Optiblot Blue (ab119211)
4. Assay Procedure

The following protocol is suited for a 6-well plate format experiment, i.e. 6 assay points, each on about $10^6$ adherent cells. If working with more cells/point or with suspension cells, the assay may be scaled up or otherwise varied as applicable except for the indicated amount of GST-PAK-CRIB and Glutahione-sepharose beads.

A. Analysis of cellular Ral-GTP levels

1. Cultivate cells in 6-well plates. Perform serum deprivation or other treatments as appropriate.

2. Prepare 6 ml Lysis solution and place on ice:

   1.2 ml 5X Lysis buffer + 4.8ml water  
   + 60 µl 100X protease inhibitors as applicable  
   + 6 µl 100 mM GDP  
   Note: Inclusion of GDP at this point quenches post-lytic GTP-loading of Rac and thus avoids false-positive signals.

   + 150 µg GST-PAK-CRIB  
   Note: Inclusion of GST-PAK-CRIB in the lysis solution quenches post-lytic GAP Ral-bound GTP and thus minimizes the loss of signal. Thaw GST-PAK-CRIB quickly in a 37°C water bath. Thawed GST-PAK-CRIB is stable for up to 3 weeks kept at 4°C.
3. After appropriate treatment/stimulation aspirate off medium from wells and lyse cells by addition of 1 ml ice-cold Lysis solution. Scrape off cell debris (with a rubber policeman) and transfer extracts to vials on ice. Vortex.

4. Clarify lysates by centrifugation (15 min, 12,000 x g, 4°C) Transfer supernatants to new vials on ice.

   Note: Samples must be processed right away and should not be frozen or stored otherwise at this stage. Freeze-thaw cycles or too extensive delay in sample processing may cause significant loss of Rac-GTP levels by denaturation or GTP hydrolysis, respectively.

5. Take sample of lysate (commonly 30-50 μl) and process for SDS-PAGE. (This represents the total Rac load)

6. Add 40 μl of (1:1) Glutathione-sepharose slurry (pre-washed and equilibrated (1:1) in 1X Lysis buffer to the clarified lysate. Incubate for 30 minutes under constant mixing (e.g. on a rotating wheel) at 4°C


8. Wash beads 1-3 times with 1 ml ice-cold 1X Lysis buffer.

   Note: The affinity purification of Rac-GTP is usually very reliable and suffers from only little false-positive signals
owing to carry over or unspecific binding of Rac-GDP to the sepharose matrix. We therefore recommend 1-2 washing steps only for most applications, in order to avoid unnecessary signal loss.

9. Drain beads well and process for SDS-PAGE analysis. For example: Add 50 μl of Laemmli SDS-PAGE loading solution containing 2 % SDS and boil the sample for 5-8 min. (This represents the Rac-GTP pulldown.)

10. Load and resolve total Rac load and Rac-GTP pulldown samples on 12 % or higher concentrated polyacrylamide gels.

Notes:

a) We recommend loading Rac-GTP pulldown and total load samples on separate gels. Moreover, the presence of GST-PAK-CRIB in large excess over the Rac proteins can seriously compromise the gel-to-membrane transfer efficiency of the latter and lead to marked signal loss.

b) We strongly recommend cutting the gel containing the resolved Rac-GTP-pulldown samples horizontally at around the molecular weight marker size of 30 kD (use pre-stained molecular size markers!). This will yield two gel pieces: the lower one contains isolated Rac proteins and should be
processed for Western blotting as described. The upper slice contains the GST-PAK-CRIB polypeptide bands and may be stained with Coomassie or other protein dye solutions to assure the presence of the affinity probe.

11. Transfer proteins from polyacrylamide gel to PVDF (or similar) blotting membrane. Block the membrane in conventional western blot wash solution containing 1-2% BSA.

12. Incubate membrane overnight in the same BSA-containing Wash-solution as above supplemented with anti-Rac1 antibody (1:1000 dilution) on a shaker at 4°C.

Notes:

Rac1 antibody solutions can be supplemented with 0.001% azide to avoid microbial growth and re-used several times.

Signals detected in the Rac-GTP pulldown represent active Rac-GTP complexes whereas the total Rac load samples include the totality of Rac complexes, i.e. Rac-GDP and Rac-GTP. Total Rac load signals should therefore ideally be of equal intensity in all points (see Data Analysis: Fig. 1 page 13).
B. Preparation of Rac-GDP and Rac-GTP-γ-S for spiking of cell extracts

Spiking of cell extracts (either at the level of step 3 or step 4 in the procedure described above) with Rac-GDP and/or Rac-GTP-γ-S serves as a control for the specificity of the affinity purification (see Data Analysis: Fig. 2). We recommend performing this control experiment for each new application (e.g. when performing the Rac activation assay on a novel cell type).

Preparation of Rac-nucleotide complexes takes only about 15 min. If kept on ice, Rac-GDP and Rac-GTP-γ-S complexes are stable for several hours.

1. To load Rac separately with GDP and GTP-γ-S dilute 2 μg Rac1ΔC in 50 μl NLS. Mix well and split into 2 x 25 μl in two fresh vials.
   Note: *The concentration of Rac1ΔC protein provided in the kit varies depending on the preparation batch.*

2. Add 2 μl 10 mM GDP or 10 mM GTP-γ-S separately to each of the samples. Mix well and briefly spin to collect liquid on the bottom of the tubes. Incubate for 10 minutes at 37°C

3. Add 2 μl 1 M MgCl₂ and mix well. Briefly spin down liquid. Let it sit for 1 min on the bench and place on ice.
4. Add 2-10 μl each of the prepared Ral-GDP or Rac-GTP-γ-S complexes separately to cell extracts being processed in a Rac Assay either before (Section A, step 3 above) or after the centrifugation step (Section A, step 4). Note: Use extracts from unstimulated, serum-starved or otherwise treated cell points that are expected to yield little endogenous Rac-GTP.

5. Proceed with the standard Rac activation assay as described in Section A.

5. Data Analysis

Figure 1. Mouse neutrophil granulocytes were stimulated with the N-formylated peptide fMLP for the indicated time periods and subjected to a Rac-GTP pulldown. Note that the antibody provided in the kit detects specifically Rac1. See protocol for details.
Figure 2. HeLa cell extracts were spiked with 5μl of Rac1ΔC protein preloaded with GDP (D) or GTP-γ-S (T) as described above, and subjected to a Rac-GTP pulldown.
6. Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Rac-GTP signal whatsoever is detectable, not even from control cells</td>
<td>Spike cell extracts with Rac-GDP and Rac-GTP-γ-S to assure functionality of all assay components</td>
</tr>
<tr>
<td></td>
<td>Increase the number of cells/assay point</td>
</tr>
<tr>
<td></td>
<td>Use a new Rac antibody solution</td>
</tr>
<tr>
<td>No increase in Rac-GTP signal in stimulated cells as compared to control unstimulated point</td>
<td>Use a different batch of agonist/stimulus or check otherwise for its functionality</td>
</tr>
<tr>
<td></td>
<td>To avoid Rac-GDP-derived false positive signal in un-stimulated points, increase the number of washing steps for the glutathione-sepharose precipitates</td>
</tr>
</tbody>
</table>

For further technical questions please do not hesitate to contact us by email (technical@abcam.com) or phone (select “contact us” on www.abcam.com for the phone number for your region).
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