™

Rac1/cdc42 Activation Magnetic Beads Pulldown Assay

30 Assays

Catalog No. 17-10394

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

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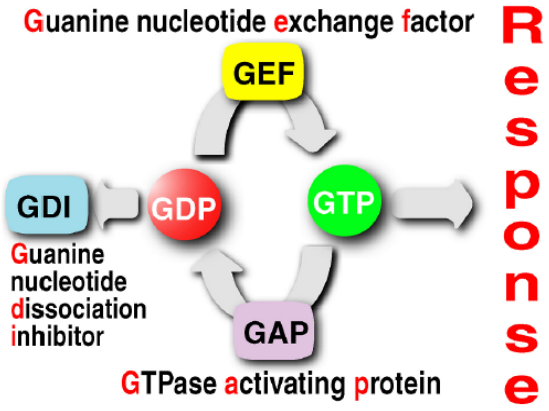
Introduction

Small GTP-binding proteins (GTPases) are important regulators of signal transduction pathways. The small GTPase acts as a key regulator of cellular functions including proliferation and differentiation and is also implicated in tumorigenesis, tumor invasion and morphogenesis.

GTPases are a family of enzymes that bind to and hydrolyze GTP, allowing them to function as molecular switches. When bound to GDP, the GTPase protein is in its inactive form. Activation is controlled by regulatory proteins called guanine nucleotide exchange factors (GEFs), which induce the release of GDP. Because GTP is present in the cell in a large excess over GDP, the resulting empty nucleotide-binding site is filled by GTP and the GTPase is activated. Another class of proteins, GTPase-activating proteins (GAPs), speed up hydrolysis of GTP to GDP, inactivating the GTPase.



The significant role of activated GTPase in tumor development makes it an important target to monitor and measure. The Active GTPase Binding Domain (PBD) is a downstream target of activated GTPase-GTP. Recombinant PBD-domain protein is provided as a fusion to GST conjugated to glutathione-magnetic beads to capture any available active GTPase-GTP within samples. Inactive GTPase-GDP will not bind to the PBD-Magnetic beads conjugate. The affinity precipitated active GTPase are detected and measured by a mouse monoclonal anti-GTPase specific antibody. An HRP conjugated secondary antibody against mouse is then added, following with immunoblot analysis.

EMD Millipore offers a panel of GTPase Activation Magnetic Beads pulldown assays to allow a convenient, efficient, and high throughput capacity analysis method for activated GTPase. The format of the kit allows for a single sample or high throughput sample analysis. Magnetic beads version pulldown assays show a higher sensitivity than agarose beads version, requiring 2 folds less cell lysate per reaction to pulldown a similar amount of active GTPase (figure 2).

Kit Components

1. Rac1/cdc42 Assay Reagent (Pak-1PBD, Magnetic beads): (Part No. CS210507) 300 µg of Pak-1 PBD in 150 µL of glutathione magnetic beads, provided as a 50% beads slurry in PBS containing 50% glycerol for a final volume of 300 µL.
2. Anti-Rac1, clone 23A8: (Part No. 05-389) One vial containing 250 µg of purified IgG2aκ in 250 µ**L** of storage buffer (0.1 M Tris-glycine, pH 7.4, 0.15 M NaCl, containing 0.05% sodium azide).
3. Anti-cdc42, mouse monoclonal IgG_1: (Part No. 05-542) One vial containing 50 µg of purified mouse IgG1 in 200 µL of 10 mM sodium phosphate, pH 7.5, 75 mM NaCl, 0.75 mM sodium azide, 0.5 mg/mL BSA and 50% glycerol.
4. Mg2+ Lysis/Wash Buffer, 5X**, (**Part No. 20-168).Two vials, each vial contains 18 mL of 125 mM, HEPES, pH 7.5, 750 mM NaCl, 5% Igepal CA-630, 50mM MgCl2, 5 mM EDTA and 10% glycerol.
5. 100X GTPγS, 10 mM**,** (Part No**.** 20-176). One vial containing 50 µL of 10 mM GTPγS, 100X stock, in sterile water. Non-hydrolyzable analog of GTP. Sufficient to label 5 mL of cell lysates.
6. 100X GDP, 100 mM**,** (Part No**.** 20-177). One vial containing 50 µL of 100 mM GDP, 100X stock, in sterile water. GDP, Guanosine 5'-Diphosphate, type I, for *in vitro* labeling of G-proteins in the inactive form. Sufficient to label 5ml of cell lysates.

Materials Not Supplied

- Stimulated and Non-stimulated Cell Lysates
- Pipettes, Micropipettes and Tips
- Electrophoresis and Immunoblotting Equipment
- Microcentrifuge
- Microcentrifuge Tubes
- Rac1 Activators
- Tissue Culture Supplies
- 4°C Tube Rocker
- Glycerol
- sodium orthovanadate
- aprotinin
- leupeptin
- sodium fluoride

Storage

Store kit materials at -20℃ for up to 4 months fro m date of receipt. Do not freeze and thaw.

Technical Information for Kit Components

Rac1/cdc42 Assay Reagent (PAK-1 PBD, magnetic beads)
Product Description: GST fusion-protein, corresponding to the p21-binding domain (PBD, residues 67-150) of human PAK-1; expressed in *E.coli*. Provided bound to glutathione agarose >90% by SDS-PAGE and Coomassie blue staining.
Specificity: Specifically binds to and precipitates Rac-GTP and cdc42-GTP from cell lysates.
Species Cross-reactivity: Human and mouse. Predicted to cross-react with all mammalian species.

Physical Form: Liquid suspension.

Anti-Rac1, clone 23A8 (mouse monoclonal IgG2b)
Immunogen: Recombinant protein containing the full length human Rac1. Clone 23A8.
Specificity: Recognizes Rac1, Mr 21 kDa. Strong Rac1 preference with slight Rac2 cross-reactivity. An additional unknown protein may be detected in some preparations, Mr 55 kDa.
Species Cross-reactivity: Human, mouse and rat, other species cross-reactivity unknown.
Physical Form: Frozen solution.

Anti-cdc42 (mouse monoclonal IgG_1)
Immunogen: Fusion protein corresponding to residues 1-191 of full-length human cdc42.
Specificity: Recognizes cdc42, Mr 22kDa. No cross-reactivity with rac, ras or rho was detected.
Species Cross-reactivity: Human, mouse, rat and dog. Other species cross-reactivity unknown.
Physical Form: Liquid at -20℃.

Note: This antibody may be reused 3-5 times for immunoblot analysis. Add 0.05% sodium azide to the blocking buffer containing the diluted antibody. **Do not use sodium azides with HRP conjugated secondary antibodies.**

Assay Instructions

Affinity Precipitation/Immunoblot Protocol

Note: It is strongly recommended that the entire procedure be read thoroughly before beginning the assay.

A. Stock Solutions

1. Mg2+ Lysis/Wash Buffer, 5X (5X MLB): Dilute to1X by adding 4 mL sterile, distilled water containing 10% glycerol, to each ml of 5X Mg2+ Lysis/Wash Buffer. To the diluted buffer add 10μg/mL aprotinin and 10 μg/mL leupeptin **(MLB)**. Optionally, the phosphatase inhibitors 25mM
2. sodium fluoride and 1 mM sodium orthovanadate may also be added to the buffer.
3. 100X GTPγS, 10 mM: Use 5 μL of GTPγS for GTP-labeling of 0.5 mL of cell lysate.
4. 100X GDP, 100 mM: Use 5 μL of GDP for GDP-labeling of 0.5 mL of cell lysate.
5. 0.5M EDTA, pH 8.0: Formulate using deionized water.

B. Cell Culture and Extract Preparation

Note: While EMD Millipore recommends using fresh lysates because GTP-Rac1 is quickly hydrolyzed to GDP-Rac1, frozen lysates may be used, provided that the lysates were snap frozen in liquid nitrogen and stored at or below -70℃. Performing a ll steps at 4 °C or on ice may reduce hydrolysis. Add PAK-1 PBD agarose directly to the lysate immediately after removing cellular debris and insoluble material by centrifugation. A sample of the lysates may be saved for assaying protein concentration, and protein loading may be equalized at the time of loading the gel.

Adherent Cells

1. Culture cells to approximately 85-90% confluence, stimulating Rac1 activation as desired.

2. Remove culture media, rinse twice with ice-cold Phosphate-Buffered Saline (PBS).
3. Add ice-cold **MLB** (0.5-1ml per 150 mm tissue culture plate) to rinsed cells in plates on ice.
4. Detach (and lyse) the cells from plates by scraping with a rubber policeman or cell scraper.
5. Transfer the lysates to microfuge tubes on ice. Proceed to Step 6.

Non-Adherent Cells

1. Culture cells and stimulate Rac1 activation as desired.
2. Pellet cells by gentle centrifugation (500xg), then rinse twice with ice-cold Phosphate-Buffered
1. Saline (PBS).
2. Discard supernatant, and add ice-cold **MLB** to the cell pellet (0.5-1 mL per 10^7 cells).
3. Lyse cells by repeated (4-5 times) pipetting.
4. Transfer the lysates to microfuge tubes on ice. Proceed to Step 6.
5. If nuclear lysis occurs, the extract may be very viscous (and difficult to pipette) due to released genomic DNA. DNA may be sheared by passing the lysate through a 26-gauge syringe needle 3-4 times (prior passage through larger-bore needles may be required first).
6. Clear lysates of insoluble cell debris by centrifugation (5 minutes, 14,000xg, 4°C).
7. Remove the extract (supernatant), and store aliquots on ice (for immediate use) or snap freeze in liquid nitrogen. Frozen extracts may be stored at -70°C or lower (for long term).

Proceed to Section C for positive and negative controls, or to Section D for the Rac1 pull-down assay.

C. GTPγS/GDP Loading for Positive and Negative Controls

Note: Samples that will not be loaded with GTPγS/GDP may be kept on ice during the loading of controls.

1. Aliquot 0.5 mL of each cell extract to two microfuge tubes.
2. To each tube, add 10 µL of 0.5 M **EDTA** (to 10mM, final concentration).
3. Add 5 µL of **100X GTPγS** (to 100 µM, final concentration) to one tube (positive control).
4. Add 5 µL of **100X GDP** (to 1 mM, final concentration) to a second tube (negative control).
5. Incubate the tubes for 30 minutes at 30℃ with a gitation.
6. Stop loading by placing the tubes on ice and adding 32.5 µL of 1 M $MgCl_2$ (to 60 mM final concentration).

D. Rac1 Pull-Down Assay

Note: The optimal conditions for a specific cell system may need to be determined empirically.

1. Aliquot 0.5 mL of each cell extract to a microfuge tube.
2. Add 10 µL (10 µg) of the **Rac1/cdc42 Assay Reagent (PAK-1 PBD, Magnetic beads**) per 0.5mL of cell lysate.
3. Incubate the reaction mixtures for 45 minutes at 4℃ with gentle agitation.
4. Pellet the beads by setting the tubes on a magnetic tube stand for a few seconds. The Magnetic beads complex will be pulled to the side.
5. Remove and discard the supernatant.
6. Wash the beads (add 0.5 mL **MLB**, mix gently, pellet beads, remove **MLB**) 3 times with **MLB.** Take care to minimize loss of beads when **MLB** is removed by leaning the pipette tip on the no beads side.

7. Resuspend the magnetic beads in 40 µL of 2X Laemmli reducing sample buffer and boil for 5 minutes. Pellet the beads by brief centrifugation. Addition of 2 µL of 1 M dithiothreitol prior to boiling may improve release of Rac1 or cdc42 from the beads.
8. Collect the beads by microcentrifuge pulse.

E. Western Blot and Detection

1. Mix the supernatant and the magnetic beads pellet and load 20 µL of the mixture per lane on an appropriate polyacrylamide gel. Total load two sets, one for Rac1 blot and the other for cdc42 blot.

NOTE: Adding beads to gel will not affect electrophoresis. Rac1 may re-associate with PAK-1 PBD after boiling. Loading slurry will help maintain consistent results.

2. Perform SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on the supernatant and transfer the proteins to nitrocellulose. Wash the blotted nitrocellulose twice with water.
3. Incubate the membrane in freshly prepared PBS containing 3% nonfat dry milk (Catalog # 20-200) and 0.05% Tween-20 (PBST-MLK) for 2 hours at room temperature with constant agitation.
4. Incubate the nitrocellulose with 1 µg/mL of **anti-Rac1, clone 23A8 (1:1000)**, **or anti-cdc42 (1:250) separately**, diluted in freshly prepared PBST-MLK overnight with agitation at 4°C.
5. Wash the membrane twice (5-10 minutes each) with PBS-0.05% Tween 20.
6. Incubate the nitrocellulose in the secondary reagent of choice (a goat anti-mouse HRP conjugated IgG, Catalog # 12-349, 1:5000 dilution was used) in PBS-MLK for 1.5 hours at room temperature with agitation.
7. Wash the membrane 3 times (5-10 minutes each) with PBS-0.05% Tween 20.
8. Use a detection method of choice. HRP conjugates are indicated for the secondary antibody, so an appropriate luminol-based chemiluminescent HRP substrate solution is recommended (colorimetric detection may be used, but sensitivity will be reduced, as compared with a chemiluminescent detection).

F. Abbreviated Procedure

Note: This abbreviated procedure is provided as a convenience to outline and facilitate performance of the assay. It is recommended that a photocopy of the page be used. As a step is completed, it may be checked off in the appropriate box. After completion of the assay, the page can then be incorporated into your lab notebook.

Cell Culture and Extract Preparation

Adherent Cells

1. Culture cells, stimulate Rac1 activation, and then rinse twice with ice-cold PBS.
2. Add ice-cold **MLB** (0.5-1 mL per 150 mm tissue culture plate).
3. Detach and lyse cells by scraping, transfer lysates to microfuge tubes on ice, then proceed to Step 4.

Non-Adherent Cells

1. Culture cells, stimulate Rac1 activation, pellet cells, and then rinse twice with ice-cold PBS.
2. Discard supernatant, and add ice-cold **MLB** to the cell pellet (0.5-1 mL per 10^7 cells).

3. Lyse cells by repeated pipetting, transfer lysates to microfuge tubes on ice, then proceed to Step 4.
4. If nuclear lysis occurs, shear DNA with a 26-gauge syringe needle. Clarify lysates by centrifugation (5 minutes, 14,000xg, 4°C).
5. Remove the extract, and store aliquots on ice or snap freeze (and store at -70°C or lower).

GTPγS/GDP Loading for Positive and Negative Controls

1. Aliquot 0.5 mL of each cell extract to two microfuge tubes.
2. Add 10 µL of 0.5 M **EDTA**.
3. Add 5 µL of **100X GTPγS** to the positive control tube.
4. Add 5 µL of **100X GDP** to the negative control tube.
5. Incubate for 30 minutes at 30℃.

Rac1 Pull-Down Assay

1. Aliquot 0.5 mL of each cell extract to a microfuge tube.
2. Add 10 µL (10µg) of **Rac1/Cdc42 Assay Reagent (PAK-1 PBD, Magnetic beads),** to each tube.
3. Incubate 45 minutes at 4℃.
4. Pellet beads by centrifugation (10 seconds, 14,000xg, 4℃).
5. Remove and discard the supernatant.
6. Wash the beads 3 times with **MLB**.
7. Resuspend the beads in 40 µL of 2X Laemmli buffer.
8. Add 2 µL of 1M dithiothreitol, boil for 5 minutes, then pellet by centrifugation.

Western Blot and Detection

1. Mix supernatant and beads, then load 20 µL on a polyacrylamide gel, perform SDS-PAGE, and western blot to a membrane. Total load two identical membranes.
2. Rinse the membrane twice with water.
3. Incubate the membrane 2 hours at room temperature in 3% PBST-MLK.
4. Incubate one membrane overnight at 4°C with **anti-Rac1** (diluted to 1 µg/mL in PBST-MLK), the other membrane overnight at 4°C with **anti-cdc42** (diluted to 1 µg/mL in PBST-MLK), Wash the membrane twice (5-10 minutes each) with PBS-0.05% Tween 20.
5. Incubate the membrane 1 hour at room temperature with secondary antibody conjugate PBST-MLK.
6. Wash the membrane 3 times (5-10 minutes each) with PBS-0.05% Tween 20.
7. Use a detection method of choice.

Example Data

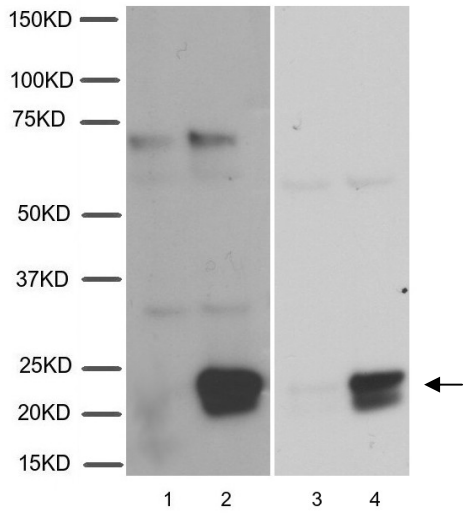
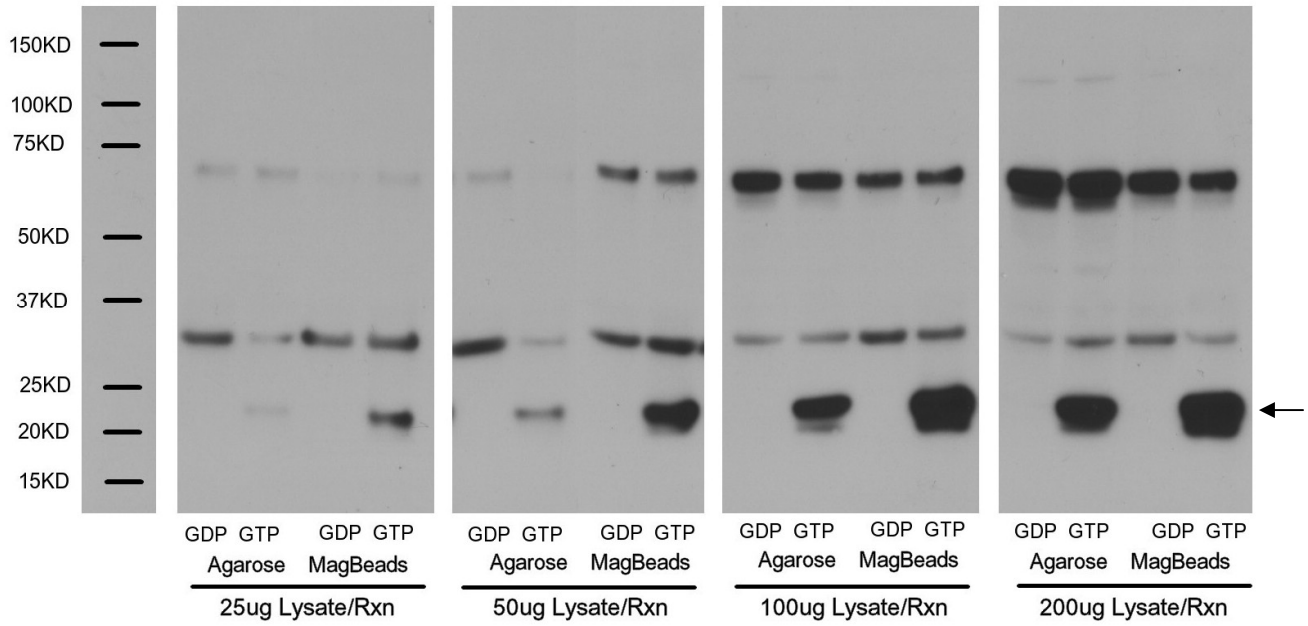
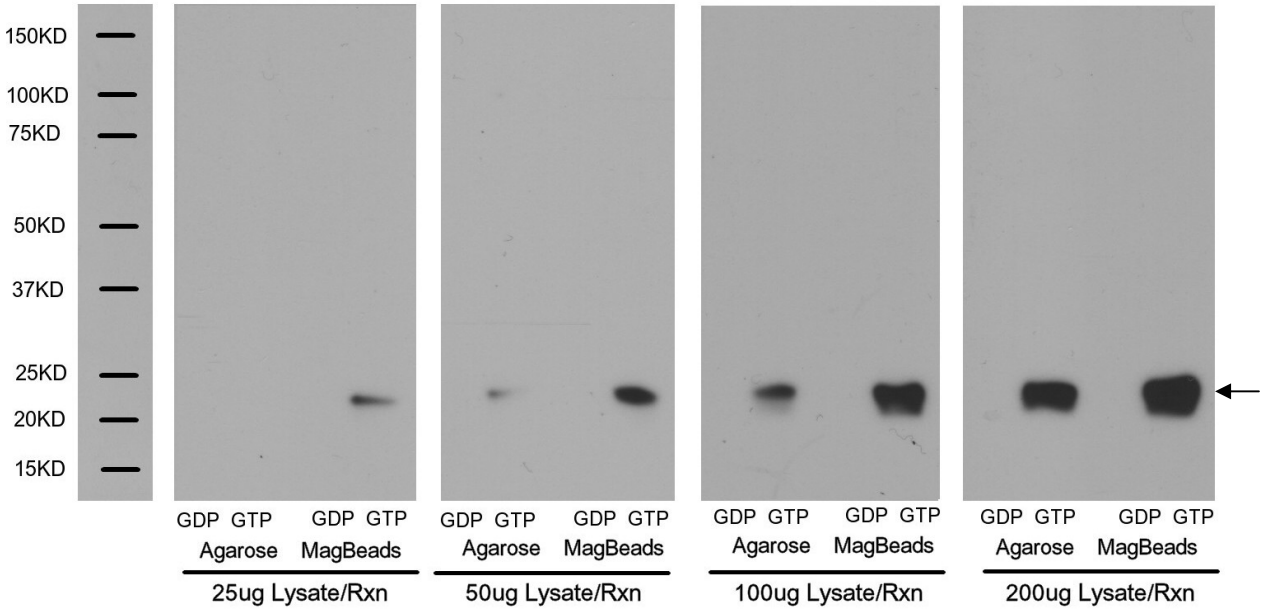


Figure 1. Affinity Precipitation/Immunoblot Analysis:
Representative blot data. NIH3T3 cell lysate treated with GDP (lane 1&3) or GTP (lane 2&4) were incubated with PAK-1 PBD Magnetic beads conjugate for 30 minutes at 4°C. The beads were then boiled in reducing sample buffer, resolved by electrophoresis, transferred to nitrocellulose and probed with a monoclonal anti-Rac1 (1 µg/mL) or anti-cdc42 (1µg/mL). Proteins were visualized using a goat anti-mouse secondary antibody conjugated to HRP and a chemiluminescence detection system. Arrow indicates Rac1 and cdc42 (~21 kDa).

A





B

Figure 2. Comparison of Magnetic Pulldown Assay over Agarose by Cell Lysate Amount Titration: Representative blot data. Various amount of NIH3T3 cell lysate treated with GDP or GTP were incubated with PAK-1 PBD Magnetic beads conjugate for 30 minutes at 4°C. The beads were then boiled in reducing sample buffer, resolved by electrophoresis, transferred to nitrocellulose and probed with a monoclonal anti-Rac1 (1 µg/mL) in A or anti-cdc42 (1µg/mL) in B. Proteins were visualized using a goat anti-mouse secondary antibody conjugated to HRP and a chemiluminescence detection system. Arrow indicates Rac1 (A) and cdc42 (B) (~21 kDa).

References

Application References:

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2. Cook, J. A., *et al*, J. Biol. Chem. **278**: 35812-35818, 2003.
3. Benard, V., *et al.,* J. Biol. Chem. **274:** 13198-13204, 1999.

General References:

4. Taylor, S.J. and D. Shalloway, Current Biology **6:** 1621-1627, 1996.

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