Certificate of Analysis

Rac1/Cdc42 Activation Assay Kit

(non-radioactive)
Catalog # 17-441
Lot # XXXXXX

Kit Components

Rac/cdc42 Assay Reagent (PAK-1 PBD, agarose), Catalog # 14-325, see page two for more information. One vial containing **300 µg** of PAK-1 PBD bound to **150 µL** of glutathione agarose beads, provided as a 50% slurry in 20mM PBS, pH 7.4, containing 50% glycerol for a final volume of **300 µL**.

Anti-Rac1, clone 23A8, Catalog # 05-389, see page two for more information. One vial containing **250 µg** of protein G purified mouse IgG_{2b} in **250 µL** of storage buffer (0.1 M Tris-glycine, pH 7.4, 0.15 M NaCl, containing 0.05% sodium azide).

Anti-cdc42, (mouse monoclonal IgG_1) Catalog # 05-542, see page two for more information. One vial containing **50 µg** of purified mouse IgG_1 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.

Mg^{2+} Lysis/Wash Buffer, 5X, Catalog # 20-168, Two vials, each vial containing **18 mL** of 5X MLB: 125 mM HEPES, pH 7.5, 750 mM NaCl, 5% Igepal CA-630, 50 mM $MgCl_2$, 5 mM EDTA and 10% glycerol.

100X GTPγS, 10 mM, Catalog # 20-176, One vial containing **50 µL** of 10 mM GTPγS, 100X stock, in 50 mM Tris-HCl, pH 7.8. Non-hydrolyzable analog of GTP. Sufficient to label 5 mL5ml of cell lysates.

100X GDP, 100mM, Catalog # 20-177, One vial containing **50 µL** of 100 mM GDP, 100X stock, in 50 mM Tris-HCl, pH 7.8. GDP (Guanosine 5'-Diphosphate) for *in vitro* labeling of G-proteins in the inactive form. Sufficient to label 5ml of cell lysates.

Quantity: 30 assays per kit.

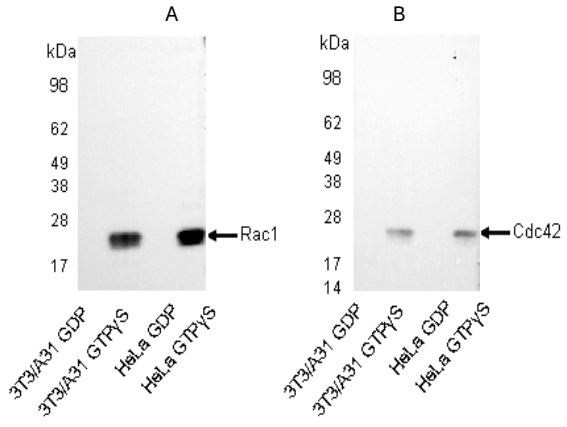
Storage and Stability: Components are stable for 1 year at -20°C from the date of shipment.

FOR RESEARCH USE ONLY; NOT FOR USE IN DIAGNOSTIC PROCEDURES. NOT FOR HUMAN OR ANIMAL CONSUMPTION

Kit Description

Quality Control Testing

The assay kit precipitated activated (GTP-bound) Rac1 and Cdc42 from 3T3/A31 and HeLa cell lysates that had been loaded with GTPγS following the protocol as described in the COA. The precipitated Rac1-GTP was detected by immunoblot analysis using anti-Rac1 (1 µg/mL) and the precipitated cdc42-GTP was detected by immunoblot using anti-cdc42 (1 µg/mL).



Affinity Precipitation / Immunoblot Analysis:
Representative lot data.
3T3/A31 and HeLa cell lysates were incubated with either GDP and GTPγS (non-cleavable GTP). The lysates where then incubated with 10 µg of PAK-1 PBD agarose to bind either activated Rac-1 (Rac1-GTP) or activated Cdc42 (Cdc42-GTP). The proteins bound to PAK-1 PBD were separated by SDS-PAGE, transferred to PVDF and probed with either anti-Rac1 (Figure A) or anti-Cdc42 (Figure B) mouse monoclonal antibodies. The arrow indicates Rac1-GTP and Cdc42-GTP, respectively.

Application Reference:

1. Cook, J. A., *et al*, J. Biol. Chem. **278**: 35812-35818, 2003.
2. Wei, Q. and Adelstein, R. S., Mol. Biol. Cell **13:** 683-697, 2002.
3. Benard, V., *et al.,* J. Biol. Chem. **274:** 13198-13204, 1999.

Reference on Methodology:

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

Technical Information for Kit Components

Rac/cdc42 Assay Reagent (PAK-1 PBD, agarose)

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 IgG_{2b})

Immunogen: Recombinant protein containing the full length human Rac1. Clone 23A8.

Specificity: Recognizes Rac1, ~21 kDa. Strong Rac1 preference with slight Rac2 cross-reactivity. Additional unknown proteins may be detected in some preparations around 55 kDa and 30 kDa.

Species Cross-reactivity: Human, mouse and rat. Other species cross-reactivity unknown.

Physical Form: Frozen solution.

Note: The dilution of this antibody prepared for immunoblot analysis may be reused 3-5 times. Add 0.05% sodium azide to the dilution to prevent microbial contamination. Do not use sodium azide with any other buffers if an HRP conjugated secondary antibody is used for detection.

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°C.

Note: The dilution of this antibody prepared for immunoblot analysis may be reused 3-5 times. Add 0.05% sodium azide to the dilution to prevent microbial contamination. Do not use sodium azide with any other buffers if an HRP conjugated secondary antibody is used for detection.

Other components required but not included as part of kit are:

- stimulated and non-stimulated cell lysates
- pipettes, pipettes, tips, or tissue culture supplies
- electrophoresis and immunoblotting equipment
- microcentrifuge and tubes
- Rac activators
- glutathione agarose
- 4°C tube rocker
- 30°C incubator or water bath
- glycerol
- 500 mM EDTA

Affinity Precipitation/Immunoblot Protocol

A. Stock Solutions

Mg^{2+} Lysis/Wash Buffer, 5X (5X MLB): Dilute to 1X by adding 4 mL sterile, distilled water containing 10% glycerol to each ml of 5X MLB used. To the diluted buffer add 10 μg/mL leupeptin and 10 μg/mL aprotinin **(MLB)**. Optionally, the phosphatase inhibitors, sodium fluoride (1 mM) and sodium orthovanadate (1 mM) may be added.

100X GTPγS, 10 mM: Use 5 μL of GTPγS for GTP-labeling of 0.5 mL of cell lysate.

100X GDP, 100 mM: Use 5 μL of GDP for GDP-labeling of 0.5 mL of cell lysate.

0.5 M EDTA, pH 8.0: Formulate using sterile, distilled water.

B. Cell Culture and Extract Preparation

Note: While Millipore recommends using fresh lysates because GTP-Rac is quickly hydrolyzed to GDP-Rac, frozen lysates may be used, provided that the lysates were snap frozen in liquid nitrogen and stored at or below -70°C. Performing all steps at 4°C or on ice may reduce hydrolysis. Add PAK-1 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 75-80% confluence, reduce serum in culture overnight and treat with a Rac activator of choice (e.g. fMLP, PMA).
2. Remove culture media, rinse twice with ice-cold Tris-Buffered Saline (TBS).
3. Add ice-cold **MLB** (0.4-0.8 mL per 100 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 Rac activation as desired.
2. Pellet cells by gentle centrifugation (500xg), then rinse twice with ice-cold Tris-Buffered Saline (TBS).
3. Discard supernatant, and add ice-cold **MLB** to the cell pellet (0.5-1 mL per 10^7 cells).
4. Lyse cells by repeated (4-5 times) pipetting.
5. Transfer the lysates to microfuge tubes on ice. Proceed to Step 6.

6. 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).
7. Preclear the cell lysate by adding 100 μL of glutathione agarose per 1 mL of lysate and rock for 10 minutes at 4°C. Collect the agarose beads by pulsing for 5 seconds in the microcentrifuge at 14,000 x g.
8. Remove the 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). Save a sample of the lysates for protein quantification (see note above). **Proceed to Section C for positive and negative controls, or to Section D for the Rac pull-down assay.**

C. *In vitro* GTPγS/GDP Protein Loading for positive and negative controls

Note: *In vivo* stimulation of cells will activate approximately 10% of the available Rac, whereas *in vitro* GTPγS protein loading will activate nearly 100% of the Rac that can be activated.

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 10 mM 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 the second tube (negative control).
5. Incubate the tubes at 30°C for 15 minutes with agitation.

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 lysate to a microfuge tube.
2. Immediately add 10 μL (10 μg) of **Rac/cdc42 Assay Reagent (PAK-1 PBD, agarose)** per 0.5 mL of cell lysate.
3. Gently rock the reaction mixture at 4°C for 60 minutes (30 minutes for GTPγS and GDP loaded lysates).
4. Collect the agarose beads by pulsing for 5 seconds in the microcentrifuge at 14,000xg.
5. Remove and discard the supernatant.
6. Wash the beads 3 times with 0.5 mL of **MLB** (add 0.5 mL **MLB**, mix gently, pellet beads, remove **MLB**).
7. Resuspend the agarose beads in 40 μL of 2X Laemmli reducing sample buffer and boil for 5 minutes. Collect the beads by microcentrifuge pulse.

E. Western Blot and Detection

1. Mix the supernatant and the agarose pellet and load 20 μL of the mixture per lane on an appropriate polyacrylamide gel.
   NOTE: Adding beads to gel will not affect electrophoresis. Rac 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 PVDF. Wash the blotted PVDF twice with water.
3. Block the blotted PVDF in freshly prepared 3% nonfat dry milk (Catalog # 20-200) in PBS (PBS-MLK) for 30 minutes at room temperature with constant agitation.
4. Incubate the PVDF with 1 μg/mL of **anti-Rac1**, diluted in freshly prepared PBS-MLK overnight with agitation at 4°C.
5. Wash the PVDF twice with water.
6. Incubate the PVDF 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 PVDF with water twice (5-10 minutes for each wash).
8. Wash the PVDF in PBS-0.05% Tween 20 for 3-5 minutes.
9. Rinse the PVDF in 4-5 changes of water.
10. Use 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 this 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 Rac activation, and then rinse twice with ice-cold TBS.
- ☐ 2. Add ice-cold **MLB** (0.4-0.8 ml per 100 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 Rac activation, pellet cells, and then rinse twice with ice-cold TBS.
- ☐ 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.
- ☐ 5. Clarify lysates with 100 µL of glutathione agarose per 1 ml lysate (10 minutes, 4°C) and collect beads with a centrifuge pulse.
- ☐ 6. Remove the supernatant, 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 15 minutes at 30°C.

Rac1 Pull-Down Assay

- ☐ 1. Aliquot 0.5 ml of each cell extract to a microfuge tube.
- ☐ 2. Add 10 µL (10 µg) of **Rac/cdc42 Assay Reagent (PAK-1 PBD, agarose)** to each tube.
- ☐ 3. Incubate 60 minutes at 4°C (30 minutes for GTPγS and GDP loaded lysates).
- ☐ 4. Pellet beads by centrifugation (5 seconds, 14,000xg, 4°C).
- ☐ 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.

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.
- ☐ 2. Rinse the membrane twice with water.
- ☐ 3. Incubate the membrane 30 minutes at room temperature in 3% PBS-MLK.
- ☐ 4. Incubate the membrane overnight at 4°C with **anti-Rac1** or **anti-Cdc42** (diluted to 1 µg/mL in PBS-MLK).
- ☐ 5. Wash the membrane twice (5-10 minutes each) with water.
- ☐ 6. Incubate the membrane 1.5 hours at room temperature with secondary antibody conjugate in PBS-MLK.
- ☐ 7. Wash the membrane twice (5-10 minutes each) with water.
- ☐ 8. Wash the membrane (3-5 minutes) in PBS-0.05% Tween 20.
- ☐ 9. Rinse the membrane in 4-5 changes of water.
- ☐ 10. Use a detection method of choice.