Product Manual

Rac1 Activation Kit

Catalog Number: ADI-EKS-450

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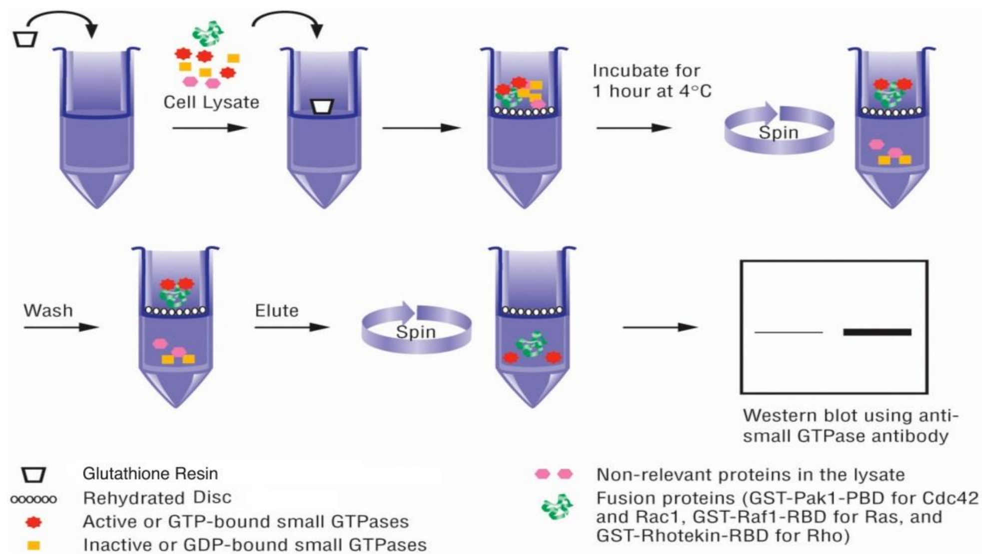
INTRODUCTION

The Rac1 Activation Kit provides a simple, convenient and efficient tool for monitoring the activation of the small GTPase, Rac1. This assay uses a GST-fusion protein containing the p21-binding domain (PBD) of human p21-activated kinase 1 (Pak1) to affinity precipitate active Rac1 (GTP-Rac1) from cell lysates. The GST-Pak-PBD fusion protein (~35kDa) is incubated with cell lysate and an Immobilized Glutathione Disc. The pulled-down active or GTP-Rac1 is detected by Western blot analysis using a specific Rac1 Antibody.



Rac, cdc42 and the Rho subfamilies belong to the Rho-family of small GTP-binding proteins (G proteins or GTPases), which have been implicated in regulating a variety of cellular functions including actin cytoskeleton organization, cell growth control and development, transcriptional activation, membrane trafficking, and cell transformation[1]. The mammalian Rho GTPase family currently consists of three subfamilies, Rho (RhoA, -B, -C), Rac (Rac1, -2, -3), and cdc42 (Cdc42Hs, G25k). Cdc42 promotes actin polymerization to form filopodia, which are found primarily in motile cells and neuronal growth cones, whereas Rac induces the formation of lamellipodia and membrane ruffles[1]. Cdc42/Rac are important upstream regulators of

the protein kinase cascade that controls the SAPK/JNK and p38 activity and there is emerging evidence that SAPK/JNK pathway is involved in mediating cell death following exposure to stress factors.

Interestingly, cdc42/Rac has been shown to be increased in select neuronal populations in cases of AD (Alzheimer's disease) in comparison to age-matched controls. This suggests the significance of cdc42/Rac in relation to the pathogenic process and contribution to neuronal degeneration[(2)].

Like all small GTPases, cdc42 and Rac act as switches regulating molecular events by cycling between an inactive GDP-bound state and an active GTP-bound state. Activation and inactivation of Cdc42/Rac is regulated by GEPs (guanine nucleotide exchange factors) and GAPs (GTPase activating proteins), respectively. Cdc42/Rac are further regulated by GDIs (guanine nucleotide inhibitors), which can inhibit both the exchange of GTP and hydrolysis of bound GTP[(1,3)].

Activated cdc42/Rac (GTP-bound forms) interact with a variety of downstream effectors, including p21-activated kinase 1 (Pak1). Binding of cdc42/Rac to the p21-binding domain (PBD) from cdc42/Rac inhibits intrinsic and GAP-enhanced GTPase activity of cdc/Rac. Therefore, the PBD of Pak1 can be used as a probe to specifically isolate active or GTP forms of cdc42 and Rac.

PRECAUTIONS

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- Please read the complete kit insert before performing this assay.

MATERIALS SUPPLIED

Rac1 Activation Kit contains the following components in sufficient quantities for 30 pull-down assays.

PART#	COMPONENT	SIZE	DESCRIPTION
80-2047	Glutathione Resin	35 each	50% slurry containing 0.05% sodium azide. **Store at 4°C.**
80-1743	2X SDS Sample Buffer	1.5 mL	Sample loading buffer containing Tris-HCl, pH 6.8, 4% SDS (w/v), 2% glycerol and 0.05% bromophenol blue. **Store at 4°C.**
80-1742	1X Lysis/Binding/Wash Buffer	100 mL	Tris based buffer used for cell lysis, protein binding and washing. **Store at 4°C.**
80-1746	Rac1 Antibody	50 µL (5 units)	Mouse monoclonal IgG_{2b}. Antibody reacts with Rac1 from human, mouse, rat, dog, and chicken. One unit of antibody is defined as the amount of antibody required to detect Rac1 in 20 µg of NIH/3T3 cell lysates by Western blotting (8.5 x 7.5cm membrane). **Store at -20°C.**
80-1745	GST-Pak1-PBD	600 µg	1-2 mg/mL GST-fusion protein containing the PBD of Pak1. GST-Pak1-PBD interacts with Rac1 from human, mouse and possibly from all mammalian species. **Store at -70°C.**
80-1741	100X GDP	50 µL	100 mM Guanosine 5'-Diphosphate in sterile water. **Store at -70°C.**

80-1740	100X GTPγS	50 µL	10 mM Guanosine 5'-O-(3-thiophosphate), a non-hydrolysable analog of GTP, in sterile water. **Store at -70°C.**
80-1739	Spin Cups	30 each	
80-1738	Collection Tubes	90 each	

STORAGE

Upon receipt, store individual components as indicated, until the kits expiry date.

OTHER MATERIALS NEEDED BUT NOT SUPPLIED

- Protease Inhibitors
- Tris Buffered Saline (TBS: 25 mM Tris-HCl, pH 7.5, 150 mM NaCl)
- 0.5 M EDTA, pH 8.0
- 1 M $MgCl_2$
- Variable speed bench-top microcentrifuge
- β-mercaptoethanol
- Electrophresis apparatus
- Polyacrylamide gel, 12% or 4-20%
- Nitrocellulose or PVDF membrane
- BSA, Fraction V
- Tween-20
- Nonfat dry milk
- NaN_3
- Anti-mouse IgG horsereadish peroxidase conjugated antibody
- Chemiluminescent Substrate
- X-ray film or CCD camera

CRITICAL PARAMETERS AND NOTES

- GTP-Rac1 is quickly hydrolyzed to GDP-Rac1. It is best to use fresh lysate for each assay.
- Use from 500 µg to 1 mg of total lysate per assay, for optimal pilot experiments.
- The components in each kit lot # have been quality assured and warranted in this specific combination only; please do not mix them with components from other kit lot #s. It is recommended that the reagents are not used beyond the kit expiration date.

SAMPLE PREPARATION

***NOTE**: Add protease inhibitors to Lysis/Binding/Wash Buffer before use (eg. 1 μg/mL aprotinin, 1μg/mL leupeptin and 1mM PMSF).*

Cell preparation for Adherent Cells

a. After treatment, carefully remove the medium and rinse the cells once with ice-cold TBS.

b. Add 0.5-1.0 mL **Lysis/Binding/Wash Buffer** per 75 cm^2 flask (~1-2 x 10^7 cells) or 0.3-0.5 mL **Lysis/Binding/Wash Buffer** per 100 mm plate with cells at 80-90% confluency.

c. Scrape the cells and transfer to a microcentrifuge tube. Vortex the tube briefly and incubate on ice for 5 minutes.

d. Centrifuge at 16,000 x *g* at 4°C for 15 minutes.

e. Transfer the supernatant (total lysate) to a new tube.

Cell preparation for Non-adherent Cells

a. Pellet the cells from one 75cm^2 flask (~1-2 x 10^7 cells) at 100 x *g* for 5 minutes. Resuspend the cells in 10 mL ice-cold TBS.

b. Pellet the cells at 100 x *g* for 5 minutes and carefully remove TBS.

c. Add 0.5-1.0 mL **Lysis/Binding/Wash Buffer** to the cell pellet and resuspend the pellet.

d. Transfer the sample to the microcentrifuge tube and incubate on ice for 5 minutes.

e. Centrifuge at 16,000 x *g* at 4°C for 15 minutes.

f. Transfer the supernatant (total lysate) to a new tube.

ASSAY PROCEDURE

1. *In vitro* GTPγS or GDP Control Treatment *(Optional)*

NOTE*: The following control treatments should be performed to ensure the pull-down procedures are working properly.*

NOTE*: Use 500 µg of cell lysate for each treatment.*

a. For 500 µL lysate, add 10 µL 0.5M EDTA, pH 8.0, for a final concentration of 10 mM. Vortex the sample.

b. Add 5 µL of 10 mM **GTPγS**, for a final concentration of 0.1 mM, or 5 µL 100 mM **GDP**, for a final concentration of 1 mM. Vortex the sample.

c. Incubate the mixture at 30°C for 15 minutes with constant agitation.

d. Stop the reaction by placing the sample on ice and adding 32 µL of 1 M $MgCl_2$, for a final concentration of 60 mM. Vortex the sample.

2. Precipitation of Active Rac1

NOTE: *The following steps should be performed on ice.*

a. Place a spin cup into a collection tube for each sample.

NOTE: *The maximal volume for the spin cup is 850 µL.*

b. Swirl the bottle of **Immobilized Glutathione Resin** to thoroughly resuspend the agarose beads. Add 100 µL of 50% resin slurry to the spin cup with collection tube. Centrifuge the tubes at 6000 x g for 10-30 seconds.

c. Discard the flow through. Add 400 µL of **Lysis/Binding/Wash Buffer** to each tube with resin. Invert gently several times. Centrifuge tubes at 6000 x g for 10-30 seconds. Discard flow through.

d. Slowly thaw **GST-Pak1-PBD** on ice and immediately aliquot. Store aliquots to be used at a later date at –70°C.
e. Add 20 µg of **GST-Pak-PBD** to the spin cup containing the disc.
f. Immediately transfer up to 700 µL of the cell lysate (containing at least 500 µg of total protein) to the spin cup. Close the cap and vortex the sample.
g. Seal the cap of the collection tube with laboratory film to prevent leakage from the cap, which may result from the high concentration of detergent. Vortex the sample.
h. Incubate the reaction mixture at 4°C for 1 hour with gentle rocking.
i. Centrifuge the spin cup with collection tube at 7,200 x *g* for 10-30 seconds.
j. Remove the laboratory film and transfer the spin cup to a new collection tube.
k. Wash the resin by adding 400 µL of **Lysis/Binding/Wash Buffer** to the resin, inverting the sample three times, and centrifuging at 7,200 x *g* for 10-30 seconds. Decant the buffer. Repeat the wash two additional times.
l. Transfer the spin cup into a new collection tube.
m. Prepare 50 µL sample buffer for each pull-down assay by mixing 1 part β-mercaptoethanol to 20 parts **2X SDS Sample Buffer** (eg. mix 2.5 µL of β-mercaptoethanol to 50 µL of **2X SDS Sample Buffer**).
n. Add 50 µL **2X SDS Sample Buffer** containing β–mercaptoethanol to the resin. Vortex the sample and incubate at room temperature for 2 minutes.
o. Centrifuge at 6000 x g for 2 minutes. Remove and discard the spin cup containing the resin.
p. Boil the samples at 95-100°C for 5 minutes.
q. Centrfuge at 7,200 x *g* for 2 minutes. Samples may be electrophoreses on a gel or stored at –20°C until use.

r. Apply at least 25 µL per lane for a 10 x 10 cm mini-gel (12% or 4-20%).

3. Western Blot Analysis

NOTE: *To verify that the Western blot analysis is functioning properly, include unfractionated cell lysate as a control.*

a. Separate the proteins on SDS-PAGE and transfer to nitrocellulose or PVDF membrane.

   NOTE*: The following steps should be performed using constant agitation.*

b. Block the membrane in TBS containing 3% BSA at room temperature for 1-2 hours.

c. Rinse membrane with TBST (TBS containing 0.05% Tween-20) for 5 minutes.

d. Prepare a solution containing **Rac1 Antibody** (1:1,000 dilution) in 3% BSA and 0.1% NaN_3 in TBST. An example of a 1:1,000 dilution is to add 10 µL of the stock antibody solution to 10 mL of buffer.

e. Incubate the membrane in the Rac1 antibody solution at 4°C overnight.

   NOTE: *If the number of pull-down assays per blot is expected to be low, the diluted Rac1 antibody solution can be re-used up to three times with no performance loss. Store the diluted Rac1 antibody solution at 4°C for up to two months.*

f. Wash membrane 5 x 5 minutes with TBST.

g. Dilute the anti-mouse IgG: HRP conjugate in TBST containing 5% nonfat dry milk (eg. if using Goat Anti-Mouse IgG:HRP Conjugate, product# ADI-SAB-100, a 1:10,000-1:200,000 dilution may be used. Each user should determine the optimal conditions for their own particular experiment).

__NOTE:__ The dry milk should be completely dissolved in TBST (eg. mix the milk in TBST on a stir-plate for 30 minutes at room temperature), otherwise the milk residuals can cause background on the Western blot.

h. Incubate the membrane in the diluted anti-mouse IgG:HRP conjugate solution at room temperature for 1 hour.

i. Wash the membrane 5 x 5 minutes with TBST.

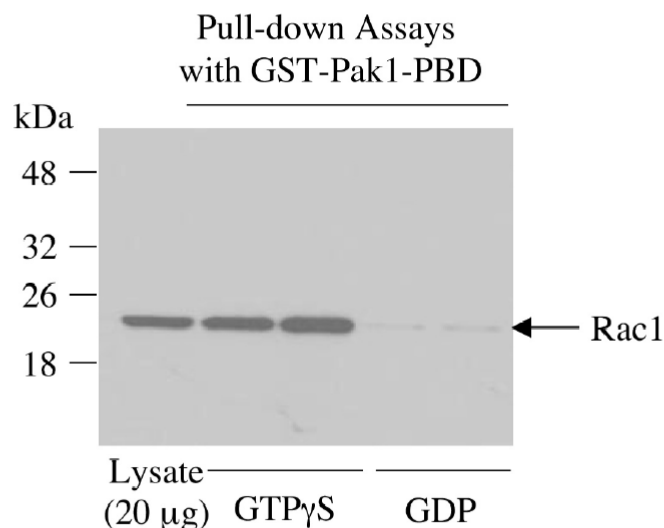
j. Incubate the membrane with chemiluminescent substrate.

k. Immediately expose the membrane to X-ray film or CCD camera.

Western Blot Analysis of Control Reactions

***NOTE**: The Rac1 signal is located at ~22kDa.*

Mouse NIH/3T3 (500 µg) cell lysates were treated *in vitro* with GTPγS or GDP to activate or inactivate Rac1. Lysates were subsequently incubated with 20 µg of GST-Pak1-PBD and an Immobilized Glutathione Disc. GTPγS-treated lysate was also incubated with GST alone in the presence of an Immobilized Glutathione Disc (negative control). Lysate and 25 µL (half the volume) of eluted samples were separated by 4-20% SDS-PAGE, transferred to a nitrocellulose membrane and probed with Rac1 Antibody.



TROUBLE SHOOTING

PROBLEM	CAUSE	SOLUTION
No activated Rac1 detected	Incorrect secondary antibody used for detection	Use goat anti-mouse IgG
	No activated Rac1 present in lysates	Include GTPγS-treated lysate as positive control for pull-down
	Insufficient activated Rac1	Increase the amount of lysate used for detection
	GST-Pak1-PBD was not added	Add GST-Pak1-PBD to the reactions
	Degraded GST-Pak1 PBD	Avoid multiple freeze/thaw cycles of GST-Pak1-PBD
	Degraded proteins	Add protease inhibitors to the Lysis/Binding/Wash Buffer before lysing cells
	Detection system is not functioning properly or requires optimization	Consult the instructions for the detection system being used
No signal with GTPγS or strong signal with GDP	GTPγS or GDP are no longer functional	Aliquot GTPγS or GDP after the first thaw and store at –20°C. Avoid repeated freeze/thaw cycles of the resuspended solution
	Incorrect concentration of EDTA or $MgCl_2$	Prepare new solutions with correct concentration
High background on Western blot	Inadequate blocking and/or washing	Optimize blocking; increase length, number or volume of washes
	Secondary antibody concentration is too high	Use the secondary antibody at a higher dilution

REFERENCES

1. Van Aelst, L. and D'Souza-Schorey, C. (1997) *Genes & Dev*. **11**: 2295-2322.
2. Zhu, X., Raina, A. K., Boux, H., Simmons, Z. I. Takeda, A. and Smith, M. A. (2000) *Int. J. Devl. Neuroscience* **18**: 433-437.
3. Takai, Y., Sasaki, T., and Matozaki, T. (2001) *Physiol. Rev*. **81**: 153-208.

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