Rap1 activation kit

Catalog Number: ADI-EKS-455

Table of Contents

ASSAY DESIGN

The Rap1 activation kit provides a simple, convenient and efficient tool for monitoring the activation of the small GTPase, Rap1. This assay uses a GST-fusion protein containing the Rap1 binding domain (RBD) of human Ra1GDS to affinity precipitate active Rap1 (GTP-Rap1) from cell lysates. The GST-Ra1GDS-RBD fusion protein (~40kDa) is incubated with cell lysate and a Glutathione Resin. The pulled-down active or GTP-Rap1 is detected by Western blot analysis using a specific Rap1 Antibody.

SCIENTIFIC OVERVIEW

Rap1, a member of the Ras superfamily, has been implicated in regulating a variety of cellular processes including proliferation, differentiation and adhesion (1). There are two main isoforms, Rap1A and 1B. Both isoforms are known to be isoprenylated at the carboxyl-terminal and phosphorylated by the cAMP-dependent protein kinase A (PKA).

Accumulating evidence indicates that Rap1 plays an important role in the regulation of a variety of integrin-dependent cellular functions. Furthermore, Rap1 has been reported to be activated by several extracellular signals and second messengers including calcium, diacylglycerol, and cAMP (2). Interestingly, dysregulation of Rap1 has been reported to play a central role in tumorigenesis (3). Like all small GTPases, Rap1 cycles between an inactive GDP-bound state and an active GTP-bound state. Activated Rap1 interacts with a variety of downstream effectors, including RalGDS. Binding of Rap1 to the Rap1-binding domain (RBD) from Ra1GDS inhibits intrinsic and GAP-enhanced GTPase activity of Rap1. Therefore, the Ra1GDS- RBD can be used as a probe to specifically isolate active or GTP-Rap1.

PRECAUTIONS

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC OR THERAPEUTIC PROCEDURES.

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

MATERIALS PROVIDED

The Rap1 activation kit contains the following components in sufficient quantities for 30 pull-down assays.

PART#	COMPONENT	SIZE	DESCRIPTION
80-2047	Glutathione Resin	3.0 mL	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.**
80-1925	Rap1 Antibody	50 µL (5 units)	Rabbit polyclonal IgG_1. Antibody reacts with Rap1A and Rap1B from human, mouse, and rat. One unit of antibody is defined as the amount of antibody required to detect Rap1 in 40µg of NIH/3T3 cell lysates by Western blotting (8.5 x 7.5cm membrane). **Store at -20°C.**
80-1926	GST-Ra1GDS-RBD	600 µg	1-2mg/mL GST-fusion protein containing the RBD of Ra1GDS. GST-Ra1GDS-RBD interacts with Rap1 from human, mouse, and rat. **Store at -70°C.**
80-1741	100X GDP	50 µL	100mM Guanosine 5'-Diphosphate in sterile water. **Store at -70°C.**
80-1740	100X GTPγS	50 µL	10mM 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.

MATERIALS REQUIRED BUT NOT PROVIDED

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

CRITICAL PARAMETERS AND NOTES

- GTP-Rap1 is quickly hydrolyzed to GDP-Rap1. It is best to use fresh lysate for each assay, if possible, otherwise freeze the lysate at –70°C immediately after precipitation.
- Use from 500µg to 1mg 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, 1 µg/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 0.5-1.0mL **Lysis/Binding/Wash Buffer** per 75cm^2 flask (~1-2 x 10^7 cells) or 0.3-0.5mL **Lysis/Binding/Wash Buffer** per 100mm plate with cells at 80-90% confluence.

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.

f. Reserve a sample of the lysate to determine protein concentration.

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 10mL ice-cold TBS.

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

c. Add 0.5-1.0mL **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.

g. Reserve a sample of the lysate to determine protein concentration.

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 500μg of cell lysate for each treatment.*

***NOTE:** Aliquot the GTPγS and GDP to minimize freeze/thaw cycles.*

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

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

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

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

2. Precipitation of Active Rap1

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

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

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

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-Ra1GDS-RBD** on ice and immediately aliquot. Store aliquots to be used at a later date at –70°C.

e. Add 20μg of **GST-Ra1GDS-RBD** to the spin cup containing the resin.

f. Immediately transfer up to 750μL of the cell lysate (containing at least 500μg 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 400µL 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 50µL sample buffer for each pull-down assay by mixing 1 part β-mercaptoethanol to 20 parts **2X SDS Sample Buffer** (eg. mix 2.5µL of β-mercaptoethanol to 50µL of **2X SDS Sample Buffer**).

n. Add 50µL **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 25µL 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 membrane.

NOTE*: Using a PVDF membrane may result in high background signal.*

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

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

NOTE*: Do not block with nonfat dry milk. Nonfat dry milk will significantly reduce the Rap1 signal on Western blot.*

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

d. Prepare a solution containing **Rap1 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 10µL of the stock antibody solution to 10mL of buffer.

e. Incubate the membrane in the Rap1 antibody solution at 4°C overnight.

NOTE: *If the number of pull-down assays per blot is expected to be low, the diluted Rap1 antibody solution can be re-used up to three times with no performance loss. Store the diluted Rap1 antibody solution at 4°C for up to two months.*

f. Wash membrane 5 x 5 minutes with TBST.

g. Dilute the anti-rabbit IgG: HRP conjugate in TBST containing 5% nonfat dry milk (eg. if using the Goat Anti-Rabbit IgG:HRP Conjugate, product # ADI-SAB-300, a 1:5,000-1:10,000 dilution may be used as a suggested starting dilution. Further dilutions may be possible. 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-rabbit IgG:HRP conjugate solution at room temperature for 1 hour.

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

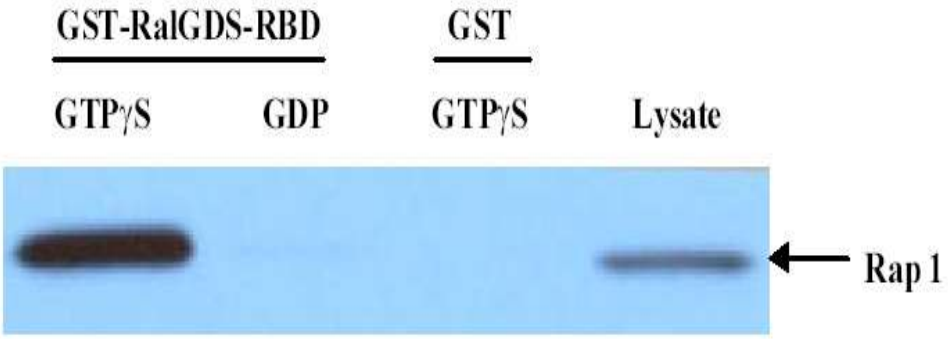
j. Incubate the membrane with chemiluminescent substrate at room temperature.

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

Western Blot Analysis of Control Reactions

NOTE*: The Rap1 signal is located at ~24kDa.*

Mouse NIH/3T3 cell lysates (500µg) were treated *in vitro* with GTPγS or GDP to activate or inactivate Rap1. Lysates were subsequently incubated with 20µg of GST-Ra1GDS-RBD 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 (30µg) and 24µL (half the volume) of eluted samples were separated by 4-20% SDS-PAGE, transferred to a nitrocellulose membrane and probed with Rap1 Antibody.



TROUBLESHOOTING GUIDE

PROBLEM	CAUSE	SOLUTION
No activated Rap1 detected	Incorrect secondary antibody used for detection	Use goat anti-rabbit IgG
	No activated Rap1 present in lysates	Include GTPγS-treated lysate as positive control for pull-down
	Insufficient activated Rap1	Increase the amount of lysate used for detection
	GST-Ra1GDS-RBD was not added	Add GST-Ra1GDS-RBD to the reactions
	Degraded GST-Ra1GDS-RBD	Avoid multiple freeze/thaw cycles of GST-Ra1GDS-RBD
	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 –70°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. Zerial, M., Huber L.A. (Ed.) Guidebook to the Small GTPases. Oxford Uni-versity Press, p 86-89.
2. Takai, Y., Sasaki, T. and Matozaki, T. (2001) *Phys. Rev*. **81**: 153-208.
3. Hattori, M. and Minato, N. (2003) *J. Biochem*. **134:** 479-84.

NOTES

Use of Product

This product contains research chemicals. As such, they should be used and handled only by or under the supervision of technically qualified individuals. This product is not intended for diagnostic or human use.

Warranty

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