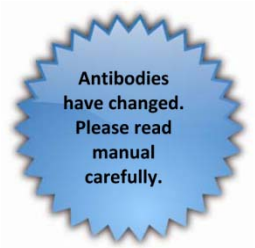

Rho activation kit

Catalog Number: ADI-EKS-465

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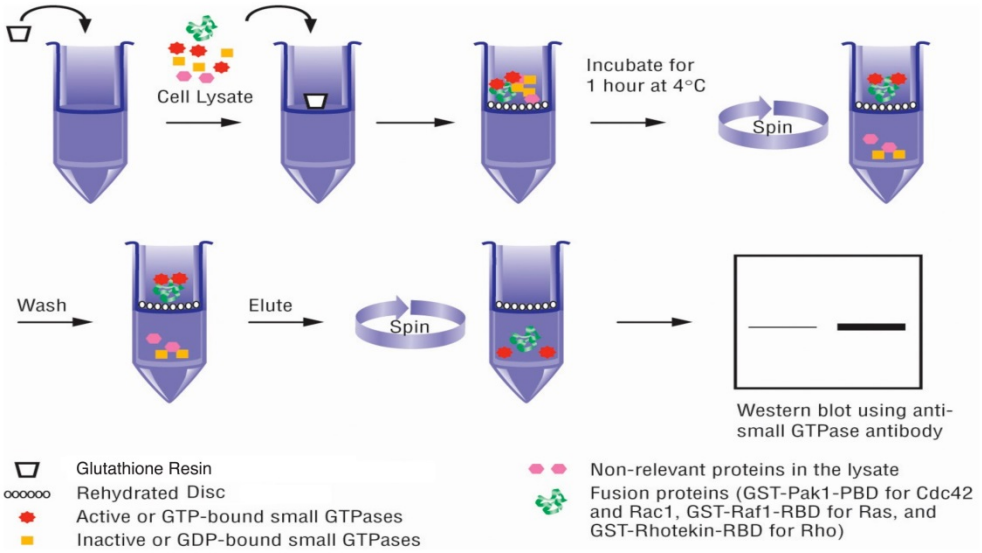
ASSAY DESIGN

The Rho activation kit provides a simple, convenient and efficient tool for monitoring the activation of the small GTPase, Rho. This assay uses a GST-fusion protein containing the Rho-binding domain (RBD) of mouse Rhotekin to affinity precipitate active Rho (GTP-Rho) from cell lysates. The GST-Rhotekin - RBD fusion protein (~36kDa) is incubated with cell lysate and an Immobilized Glutathione Disc. The pulled-down active or GTP-Rho is detected by Western blot analysis using a specific Rho Antibody.



SCIENTIFIC OVERVIEW

Rho, Cdc42 and Rac 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, Rac (Rac1, -2, -3), Cdc42 (Cdc42Hs, G25k) and Rho (RhoA, -B, -C). Rho proteins have been shown to regulate actin stress fiber formation, focal adhesion and cell migration (1). Rho has also been shown to stimulate gene transcription and participate in Ras-induced cell transformation. Increased expressions of some Rho family members appears to be a frequent event in different types of human tumors which make them potential targets for therapeutic intervention (2).

Like all small GTPases, Rho acts as a switch regulating molecular events by cycling between an inactive GDP-bound state and an active GTP-bound state. Activation and inactivation of Rho is regulated by GEPs (guanine nucleotide exchange factors) and GAPs (GTPase activating proteins), respectively. Rho is further regulated by GDIs (guanine nucleotide inhibitors), which can inhibit both the exchange of GTP and hydrolysis of bound GTP (1,3). Actived Rho (GTP-bound form) goes on to interact with a variety of downstream effectors, including Rhotekin. Binding of Rho to the Rho-binding domain (RBD) from Rhotekin inhibits intrinsic and GAP-enhanced GTPase activity of Rho. Therefore, the Rhotekin RBD can be used as a probe to specifically isolate active or GTP-Rho.

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 Rho 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	1.5mL	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	100mL	Tris based buffer used for cell lysis, protein binding and washing. **Store at 4°C.**
80-1733	Rho Antibody	75µL (5 units)	Rabbit IgG. Antibody reacts with RhoA, -B, -C from human, mouse, and rat. One unit of antibody is defined as the amount of antibody required to detect Rho in 20µg of NIH/3T3 cell lysates by Western blotting (8.5 x 7.5cm membrane). **Store at -20°C.**
80-2310	Stabilized Anti-Rabbit IgG: HRP Conjugate	2.0mL	Goat anti-Rabbit HRP conjugate
80-1732	GST-Rhotekin-RBD	3 x 4mg	5-6mg/mL GST-fusion protein containing the RBD of Rhotekin. GST-Rhotekin-RBD interacts with Rho from human, mouse and possibly from all mammalian species. **Store at -70°C.**
80-1741	100X GDP	50µL	100mM Guanosine 5'-Diphosphate in sterile water. **Store at -70°C.**
80-1740	100X GTPγS	50µL	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, 12% or 4-20%
- Nitrocellulose 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

- **Primary Antibody Change.** Earlier versions of EKS-465 used a **mouse** anti-Rho primary antibody. The kit now uses a **rabbit** anti-Rho antibody along with Stabilized Goat Anti-Rabbit IgG:HRP conjugate as the secondary antibody. When performing the Western blot step, it is best to verify that you apply the appropriate secondary antibody for the species of primary antibody used.
- GTP-Rho is quickly hydrolyzed to GDP-Rho. It is best to use fresh lysate for each assay, if possible, otherwise freeze lysate at –70°C immediately after precipitation.
- Use from 500µg to 1mg of total lysate per assay, for optimal pilot experiments.
- Use at least 1mg of total lysate per assay for measuring the activation of endogenous Rho.
- 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. 1μg/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 0.5-1.0mL **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% 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.
f. Save a sample of the lysate to determine protein concentration.

Cell Preparation for Non-Adherent Cells

a. Pellet the cells from a 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. Save 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.*

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 Rho

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 850μL.*

b. Swirl the bottle of **Glutathione Resin** to thoroughly resuspend the agrose beads. Add 100 μL of the 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 the flow through.

d. Slowly thaw **GST-Rhotekin-RBD** on ice and immediately aliquot. Store aliquots to be used at a later date at -70˚C.

e. Add 400μg of **GST-Rhotekin-RBD** to the spin cup containing the resin.

f. Immediately transfer up to 700μL 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 6,000 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 6,000 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. Centrfuge 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. . 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%).

***NOTE**: A 12% gel provides better separation of GST-Rhotekin-RBD from Rho.*

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**: For best results, use a nitrocellulose membrane. Using a PVDF membrane may result in higher 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 1-2 hours.

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

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

d. Prepare a solution containing **Rho Antibody** (1:500 dilution) in 3% BSA and 0.1% NaN_3 in TBST. An example of a 1:500 dilution is to add 20µL of the stock antibody solution to 10mL of buffer.

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

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

f. Wash membrane 5 x 5 minutes with TBST.

g. Dilute the Stabilized Anti-Rabbit IgG: HRP Conjugate by adding 20mL to 10mL (1:500 dilution) of 5% nonfat dry milk in TBST. Please see the Critical Parameters and notes section for important information concerning the primary antibody change..

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.

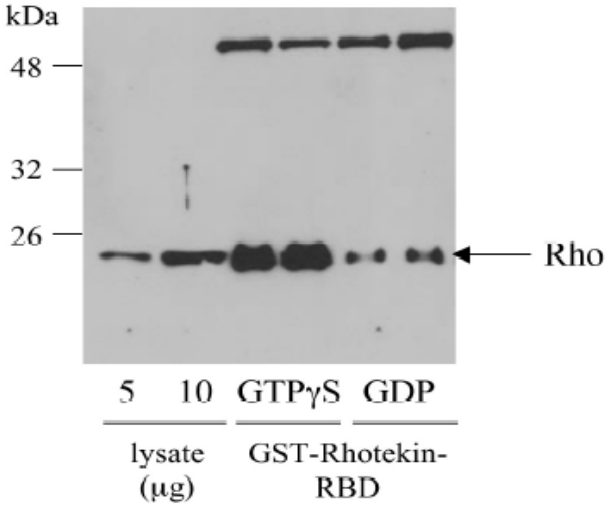
g. Incubate the membrane with chemiluminescent substrate at room temperature.

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

Western Blot Analysis of Control Reactions

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

Mouse NIH/3T3 cell lysates (500µg) were treated *in vitro* with GTPγS or GDP to activate or inactivate Rho. Lysates were subsequently incubated with GST-Rhotekin-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 (10µg) and 25µL (half the volume) of eluted samples were separated by 12% SDS-PAGE, transferred to a nitrocellulose membrane and probed with Rho Antibody.


kDa
48
32
26
Rho
5
10
GTPγS
GDP
lysate (μg)
GST-Rhotekin-RBD

TROUBLESHOOTING GUIDE

PROBLEM	CAUSE	SOLUTION
No activated Rho detected	Incorrect secondary antibody used for detection	Use goat anti-mouse IgG
	No activated Rho present in lysates	Include GTPγS-treated lysate as positive control for pull-down
	Insufficient activated Rho	Increase the amount of lysate used for detection
	GST-Rhotekin-RBD was not added	Add GST-Rhotekin-RBD to the reactions
	Degraded GST-Rhotekin PBD	Avoid multiple freeze/thaw cycles of GST-Rhotekin-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

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2. Aznar, S., Fernandez-Valeron, P., Espina, C., and Lacal, J.C. (2004) *Cancer Lett*. **206**: 181-191.
3. Takai, Y., Sasaki, T., and Matozaki, T. (2001) *Physiol. Rev*. **81**: 153-208.

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.

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