

# Ras activation kit

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OR THERAPEUTIC PROCEDURES.**



## **ASSAY DESIGN**

The Ras activation kit provides a simple, convenient and efficient tool for monitoring the activation of the small GTPase, Ras. This assay uses a GST-fusion protein containing the Ras-binding domain (RBD) of Raf1 to affinity precipitate active Ras (GTP-Ras) from cell lysates. The GST-Raf1-RBD fusion protein (~42kDa) is incubated with cell lysate and an Immobilized Glutathione Resin. The pulled-down active or GTP-Ras is detected by Western blot analysis using a specific Ras Antibody.

## **SCIENTIFIC OVERVIEW**

The 21 kDa monomeric G protein, Ras is recognized to be a major regulator of cellular growth, apoptosis, and differentiation (1-3). It is estimated that ~30% of all human cancers harbor mutations in the Ras gene, which render it bound in its active GTP bound state.

Activated Ras interacts with a variety of downstream effectors, including Raf1. Binding of Ras to the Ras -binding domain (RBD) of Raf1 inhibits intrinsic and GAP-enhanced GTPase activity of Ras. Therefore, the Raf1-RBD can be used as a probe to specifically isolate active or GTP bound Ras.

## **PRECAUTIONS**

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

## **MATERIALS PROVIDED**

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

<b>PART#</b>	<b>COMPONENT</b>	<b>SIZE</b>	<b>DESCRIPTION</b>
80-2047	Glutathione Resin	3.0 mL	50% slurry containing 0.05% sodium azide. <b>Store at 4°C.</b>
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. <b>Store at 4°C.</b>
80-1742	1X Lysis/Binding/Wash Buffer	100mL	Tris based buffer used for cell lysis, protein binding and washing. <i>Note: Buffer prepared without 1mM DTT</i> <b>Store at 4°C.</b>
80-1735	Ras Antibody	250µL (5 units)	Mouse monoclonal Ig <sub>2a</sub> κ. Antibody reacts with Ras from human, mouse, and rat. One unit of antibody is defined as the amount of antibody required to detect Ras in 20µg of NIH/3T3 cell lysates by Western blotting (8.5 x 7.5cm membrane). <b>Store at 4°C.</b>
80-1764	GST-Raf1-RBD	2.4mg	2-3mg/mL GST-fusion protein containing the RBD of Raf1. GST-Raf1-RBD interacts with Ras from human, mouse and possibly from all mammalian species. <b>Store at -70°C.</b>
80-1741	100X GDP	50µL	100mM Guanosine 5'-Diphosphate in sterile water. <b>Store at -70°C.</b>
80-1740	100X GTPγS	50µL	10mM Guanosine 5'-O-(3-thiophosphate), a non-hydrolysable analog of GTP, in sterile water. <b>Store at -70°C.</b>
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<sub>2</sub>
- Variable speed bench-top microcentrifuge
- $\beta$ -mercaptoethanol
- Electrophoresis apparatus
- Polyacrylamide gel, 12% or 4-20%
- Nitrocellulose membrane
- BSA, Fraction V
- Tween-20
- Nonfat dry milk
- NaN<sub>3</sub>
- Anti-mouse IgG horseradish peroxidase conjugated antibody
- Chemiluminescent Substrate
- X-ray film or CCD camera

## **CRITICAL PARAMETERS AND NOTES**

- GTP-Ras is quickly hydrolyzed to GDP-Ras. It is best to use fresh lysate for each assay.
- Use from 500 $\mu$ g 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 (e.g., 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<sup>2</sup> flask (~1-2 x 10<sup>7</sup> 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.

### **Cell preparation for Non-Adherent Cells**

- a. Pellet the cells from one 75cm<sup>2</sup> flask (~1-2 x 10<sup>7</sup> 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.

## ASSAY PROCEDURE

### 1. *In vitro* GTP $\gamma$ S 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 $\mu$ g of cell lysate for each treatment.

- a. For 500 $\mu$ L lysate, add 10 $\mu$ L 0.5M EDTA, pH 8.0, for a final concentration of 10mM. Vortex the sample.
- b. Add 5 $\mu$ L of 10mM **GTP $\gamma$ S**, for a final concentration of 0.1mM, or 5 $\mu$ L 100mM **GDP**, for a final concentration of 1mM. 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 $\mu$ L of 1M MgCl<sub>2</sub>, for a final concentration of 60mM. Vortex the sample.

### 2. Precipitation of Active Ras

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

- a. Save a sample of the lysates for protein quantification.
- b. Swirl the bottle of **Immobilized Glutathione Resin** to thoroughly resuspend the agarose beads
- c. Place 100 $\mu$ L of the **Immobilized Glutathione Resin** into a spin cup with a collection tube. Centrifuge the tubes at 6,000 x g for 10-30 seconds.
- d. Discard the flow through. Add 400 $\mu$ L of Lysis/Binding/Wash Buffer to each tube with resin. Invert gently several times. Centrifuge the tubes at 6,000 x g for 10-30 seconds. Discard the flow through.

*NOTE:* The maximal volume for the spin cup is 850 $\mu$ L.

- e. Slowly thaw **GST-Raf1-RBD** on ice and immediately aliquot. Store aliquots to be used at a later date at -70°C.
- f. Add 80 $\mu$ g of **GST-Raf1-RBD** to the spin cup containing the resin.
- g. Immediately transfer up to 700 $\mu$ L of the cell lysate (containing at least 500 $\mu$ g of total protein) to the spin cup. Close the cap and vortex the sample.
- h. 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.

- i. Incubate the reaction mixture at 4°C for 1 hour with gentle rocking.
- j. Centrifuge the spin cup with collection tube at 6,000 x g for 10-30 seconds.
- k. Remove the laboratory film and transfer the spin cup to a new collection tube.
- l. 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.
- m. Transfer the spin cup into a new collection tube.
- n. 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**).
- o. Add 50µL **2X SDS Sample Buffer** containing β-mercaptoethanol to the resin. Vortex the sample and incubate at room temperature for 2 minutes.
- p. Centrifuge the tube at 6,000 x g for 2 minutes. Remove and discard the spin cup containing the resin.
- q. Boil the samples at 95-100°C for 5 minutes.
- r. Samples may be electrophoreses on a gel or stored at -20°C until use.
- s. 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 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 **Ras Antibody** (1:200 dilution) in 3% BSA and 0.1% NaN<sub>3</sub> in TBST. An example of a 1:200 dilution is to add 50µL of the stock antibody solution to 10mL of buffer.
- e. Incubate the membrane in the Ras antibody solution at 4°C overnight.

*NOTE: If the number of pull-down assays per blot is expected to be low, the diluted Ras antibody solution can be re-used up to three times with no performance loss. Store the diluted Ras 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 the Goat Anti-Mouse IgG:HRP Conjugate, product# 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).

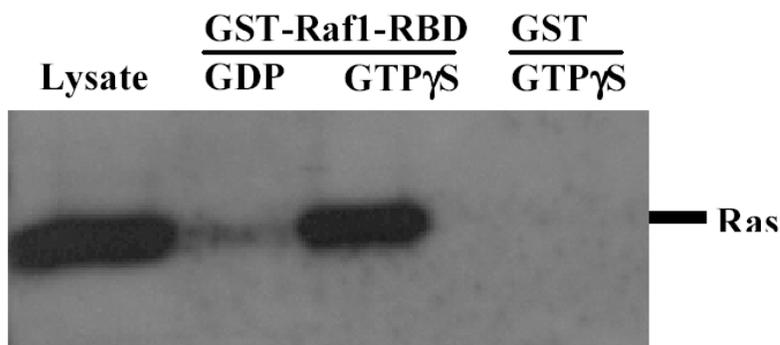
*NOTE: The dry milk should be completely dissolved in TBST (eg. mix the milk in TBST on a stir-plate for 30 minutes at room temperature), otherwise the milk residuals can cause background on the Western blot.*

- 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 Ras signal is located at ~21kDa.*

Mouse NIH/3T3 cell lysates (500µg) were treated *in vitro* with GTP $\gamma$ S or GDP to activate or inactivate Ras. Lysates were subsequently incubated with GST-Raf1-RBD and an Immobilized Glutathione Disc. GTP $\gamma$ S-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 4-20% SDS-PAGE, transferred to a nitrocellulose membrane and probed with Ras Antibody.



## TROUBLESHOOTING GUIDE

<b>PROBLEM</b>	<b>CAUSE</b>	<b>SOLUTION</b>
No activated Ras detected	Incorrect secondary antibody used for detection	Use goat anti-mouse IgG
	No activated Ras present in lysates	Include GTP $\gamma$ S-treated lysate as positive control for pull-down
	Insufficient activated Ras	Increase the amount of lysate used for detection
	GST-Raf1-RBD was not added	Add GST-Raf1-RBD to the reactions
	Degraded GST-Raf1 PBD	Avoid multiple freeze/thaw cycles of GST-Raf1-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 GTP $\gamma$ S or strong signal with GDP	GTP $\gamma$ S or GDP are no longer functional	Aliquot GTP $\gamma$ S or GDP after the first thaw and store at $-70^{\circ}\text{C}$ . Avoid repeated freeze/thaw cycles of the resuspended solution
	Incorrect concentration of EDTA or MgCl <sub>2</sub>	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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