Instruction Manual For
Ras Activation ELISA ASSAY Kit

Catalog # 17-497

Sufficient reagents for 96 Ras GTPase Activation Assays

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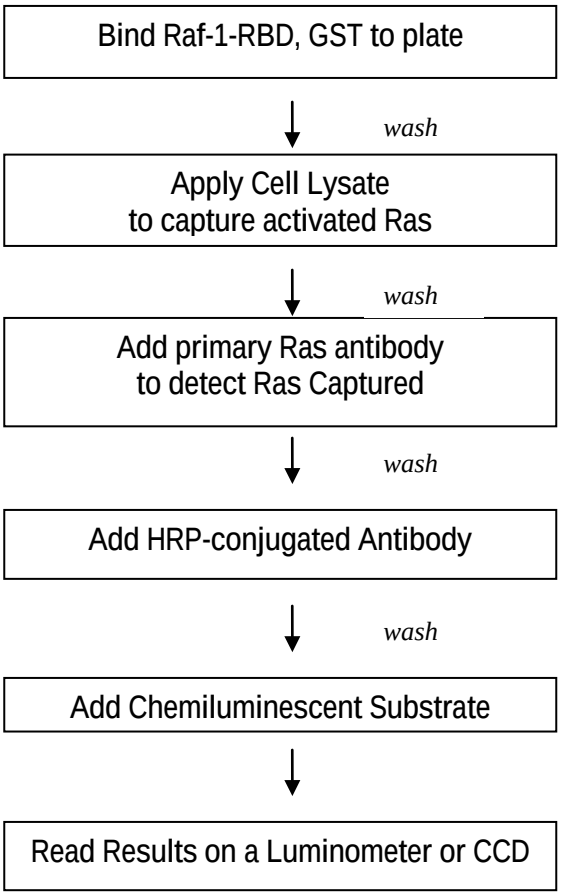
I. TEST PRINCIPLE

Ras Activation ELISA Assay Kit is designed to detect the presence of activated Ras. The assay works on the principle that Ras only binds to its downstream kinase, Raf-1 (MAP Kinase Kinase Kinase), when in its active-GTP bound state. In this state, Ras binds to a domain of Raf-1 kinase referred to as the Ras Binding Domain (RBD). Ras is unable to bind to Raf-1-RBD in its inactive/GTP bound state. A recombinant Raf-1-RBD is provided for the capture of activated Ras from your sample. Raf-1-RBD binds to the wells of a 96-well glutathione-coated ELISA plate via a GST/ Glutathione interaction, thus capturing the active Ras and allowing the inactive/GDP-bound Ras to be washed away. The captured active Ras is detected and measured quantitatively through the addition of a monoclonal anti-Ras antibody that detects K-, H-, N- Ras isoforms from human, mouse, and rat. An HRP conjugated secondary antibody is then added for the detection. Following addition of the chemiluminescent substrate, signals can be measured using a luminometer or with a CCD camera. The format of the kit allows for single sample or high throughput sample analysis.

Quantity: One Box containing the necessary reagents to perform 96 individual Ras GTPase Activation Assays. Enough cell lysate is provided to perform 8 positive controls.

Flow Chart of Process

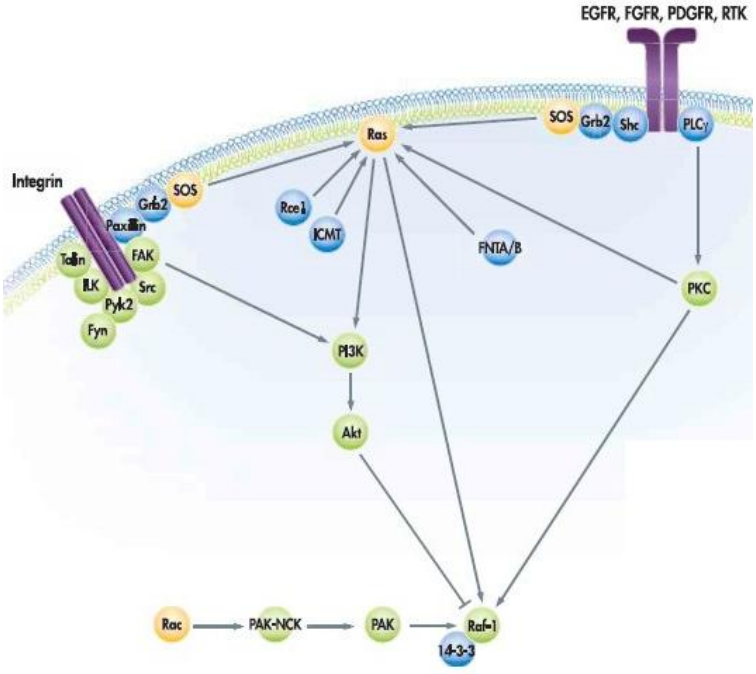


II. RAS BACKGROUND

Ras proteins function as GDP/GTP-regulated binary switches in signal transduction cascades that can lead to cell growth, proliferation, differentiation, or survival. This Ras superfamily has at least five major branches that include Ras, Rho, Ran, Arf/Sar, Rab. Ras itself has 3 primary isoforms (H-Ras, N-Ras, and K-Ras) that differ in their approximately 20 C-terminal amino acids. They are collectively referred to as Ras. In its active form, Ras is bound to GTP. This causes a conformational change that allow it to interact and bind to several effector molecules, most notably the members of the Raf family, the RalGDS family, and Phosphoinositide 3-kinases (PI3 Kinase). Ras then cleaves GTP to GDP resulting in its inactivation. In its oncogenic, mutated state, Ras is unable to hydrolyze GTP to GDP, thus staying in an active state and activating numerous pathways.

Due to its role as a key regulator of cellular functions and its implications in various cancers, Ras has been a popular target for cancer research and anti-cancer therapeutics for the past two decades for both academic and pharmaceutical research. It was also the first human oncogene identified. Mutations in cellular Ras have been found to be present in a large percentage of all human cancers. More specifically, K-Ras mutations occur frequently in lung, pancreatic, and colon cancers, where as H-Ras mutations are prevalent in bladder, kidney, and thyroid cancers, and N-Ras mutations are associated with melanoma, hepatocellular carcinoma, and leukemia (3). In recent studies, Ras activity has also been implicated in a number of human development defects such as Costello syndrome and Noonan syndrome (4).

One path that the pharmaceutical industry has taken to control Ras and its activity is by attacking what some consider its Achilles' heel. For its activation, Ras must localize to the plasma membrane, but interestingly, it lacks a transmembrane domain. To achieve this, Ras must first undergo a post-translational modification known as prenylation or geranylation at its C-terminal CAAX motif. These drugs have yet to pass clinical trials though and there is doubt that they will ever be successful in treating tumors associated with Ras activation.



III. SPECIES REACTIVITY

Species Reactivity: Human, Mouse, and Rat

IV. STORAGE AND STABILITY

Kit components arrive on dry ice, and must immediately be stored at the temperatures specified below.
Kit components are stable for 3 months from date of shipment when stored as directed.

V. KIT COMPONENTS

Store at 2-8º C

1. **Glutathione-Coated Plate, 96-well strip format:** (Part No. 3007063) 1 plate containing 12 x 8 strip-wells coated with glutathione.
2. **Anti-Ras Detection Antibody:** (Part No. 2006992) One vial containing 10 µL of anti-Ras mouse monoclonal primary antibody. Can specifically detect K-, H-, N- isoforms of Ras.
3. **Mg^{2+} Lysis/Wash Buffer, 5X:** (Part No. CS205814) 1 vial containing 18 mL of 5X Mg^{2+} Lysis/Wash Buffer for preparation of cell lysate.
4. **20% Tween®:** (Part No. # 20-246) One vial containing 3 mL of 20% Tween®.
5. **10% BSA in TBS (Blocking Buffer):** (Part No. 20-191) One bottle containing 10 mL 10% BSA in 1X TBS.
6. **20X TBS Wash:** (Part No. 20-190) One bottle containing 50 mL of 20X TBS, pH 7.4.
7. **Gt x Ms, HRP:** (Part No. CS202619) One amber vial containing 25 µL of goat anti-mouse HRP conjugated antibody.
8. **Chemiluminescent Reaction Buffer:** (Part No. 90496) One amber bottle containing 8 mL of Chemiluminescent Reaction Buffer. Keep out of light.
9. **Chemiluminescent Detection Reagent:** (Part No. 90495) One bottle vial containing 4 mL of Chemiluminescent Detection Reagent. Keep out of light.

Store at -80º C

10. **Raf-1, GST**: (Part No. 14-863) 2 vials containing 300 µg of Raf-1 at 2 µg/µL.
11. **EGF stimulated HeLa Cell Lysate:** (Part No. 12-500) 2 vials containing 40 µL of lysate at 5 mg/mL concentration, lysed in Mg^{2+} Lysis/Wash buffer (Catalog # CS202617).

Materials Not Supplied

1. Multi-channel or repeating pipettes
2. Plate shaker at room temperature and at 4^{o}C.
3. Pipettors & tips capable of accurately measuring 1-1000 µL
4. Graphing software for plotting data or graph paper for manual plotting of data
5. Multi-channel pipettor reservoirs
6. Protease inhibitors
7. Nuclease free water/ deionized water
8. PBS
9. Luminometer or CCD camera coupled imaging system

Precautions

- The Ras Activation ELISA Assay kit is designed for research use only and not recommended for internal use in humans or animals. All chemicals should be considered potentially hazardous and principles of good laboratory practice should be followed.
- The instructions provided have been designed to optimize the kit's performance. Deviation from the instructions may result in suboptimal performance of the kit and the failure to produce accurate data.

Technical Notes

- For maximum recovery of product, centrifuge original vial after fast thawing prior to removing the cap. Rapidly thaw the vial under cold water and immediately place on ice or keep at room temperature following the protocols. Aliquot the -80°C components to avoid repeated thawing and freezing. Immediately snap-freeze the vials in liquid nitrogen prior to re-storage at -80°C.
- Do not use reagents beyond the expiration date of the kit.
- Do not mix or interchange reagents from various kit lots.
- Manual Plate Washing: Vigorous washing and complete removal of all liquid by aspiration at the end of each washing step is very important to obtain low background values. Gentle agitation during the wash steps or a 2-3 minute soak may reduce background values.

VI. PREPARATION OF BUFFER/SOLUTION

1. **1X TBS:** Prepare 200 mL of 1X TBS by adding 10 mL of 20X TBS (Catalog # 20-190) to 190 mL of Milli-Q or distilled water. Store at room temperature.
2. **1X TBST (Wash Buffer)**: Prepare 800 mL of 1X TBST by adding 2 mL of 20% Tween® (v/v) (Catalog # 20-246) and 40 mL of 20X TBS (Catalog # 20-190) to 758 mL of Milli-Q or distilled water. Store at room temperature.
3. **Binding/Blocking Buffer:** For each 96 well plate, prepare 30 mL of 3% BSA in TBST by adding 10 mL of 10% BSA in TBS (Catalog # 20-191) to 20 mL of 1X TBST. This solution is stable for several days at 4°C. Discard unused portion following assay completion.
4. **1x Mg^{2+} Lysis/Wash Buffer**: Dilute 4:1 with 4 volumes deionized water to make a 1X solution. Add protease inhibitors as needed. Use immediately to lysis cells. Use excess lysis buffer to blank for quantification of cell lysate on a Bradford assay. Discard unused 1X buffer.

5. **Primary Antibody Solution**: Add 1 µL of the primary anti-Ras antibody to 9 µL of Binding/Blocking Buffer. Take 5 µL of this mix and add it to 5 mL of Binding/Blocking Buffer for a final dilution of **1:10,000**. Use directly in assay. Stable for 72 hours at 4º C.
6. **Secondary Antibody Solution**: Add 1 µL of the secondary anti-mouse HRP conjugated antibody to 5 mL of Binding/Blocking Buffer. Mix well for a final dilution of **1:5,000**. Use directly in assay, discard any unused amount.
7. **Chemiluminescent Substrate**: Prior to use, allow each component to warm separately at room temperature. Add two parts of the Chemiluminescent Reaction Buffer to one part Chemiluminescent detection reagent (2:1). Let mixture sit for approximately 1-5 minutes prior to use. Do not let reagent mixture to sit for longer as this will result in decreased signal intensity.

VII. CELL LYSATE SAMPLE PREPARATION

1. Culture cells to approximately 85-90% confluency, stimulating Ras activation as desired.
2. Remove culture media and wash cells twice ice-cold with 1X TBS (Tris Buffered Saline) or PBS (Phosphate Buffered Saline). Discard supernatant
3. Add 1X Mg^{2+}Lysis/Wash Buffer (0.5-1.0 mL per 150 mm tissue culture plate) prepared from the 5X Mg^{2+} Lysis/Wash buffer (Cat# CS202617) included in the kit by adding 10 µL of 1 µg/µL Leupeptin, 10 µL of 1 µg/µL Aprotenin, 10 µL of 1 µg/µL Pepstatin, and 100 µL of 100 mM PMSF to 9.87 mL of 1X Mg^{2+} lysis/wash buffer for a final volume of 10 mL.
4. Scrape cells from plate with a rubber policeman.
5. Transfer cells in lysis buffer to a microcentrifuge tube and incubate on ice for 15 minutes. Vortex tube for 10 seconds or sonicate briefly for 10 seconds. Centrifuge the sample at 14000 rpm for 10 minutes at 4ºC in a microcentrifuge. .
6. Collect the supernatant and calculate protein concentration using a Bradford Assay or by densitometry.
7. Keep samples cold and use immediately if not freezing away for later use. Samples can be frozen and stored at -80º C. It is suggested to use the cell lysate be immediately used or use within 6 months if storing at -80º C. Avoid repeated freeze thaws.

VIII. Ras Activity ELISA Assay Kit Protocol

1. Take out as many strips as needed to perform the assay. Store the remaining strips with descant at 4ºC. *(Suggestion, strips can be better held in place by taping down the ends of the strips onto the plate frame).*
2. Pre-rinse the glutathione coated wells that you'll be using with 200 µL of Wash Buffer (TBST). Flick out the wash, and repeat 2 more times.
3. For each well to be assayed, add **2.5 µL of 2 µg/µL Raf-1** to 47.5 µL of Binding/Blocking and add 50 µL per well. Cut plate sealer to size and cover wells to be assayed. Incubate the wells with rocking at 4ºC, for 1 hour.
4. Flick out solution. Wash each well with 200 µL of Wash Buffer and flick out the wash. Repeat 3 times.
5. **Positive control**: add **10 µL of 5 mg/mL EGF stimulated HeLa cell lysate to 40 µL** of Binding/Blocking Buffer. **Negative control** wells can be prepared by adding only 50-200 µL of Binding/Blocking buffer to a well coated with Raf-1 or by adding 50 µg Ras negative cell line (293 –VE) cell lysate (not included) per well or by adding 50 µg of HeLa cell extract treated with GDP (see section VI), to inactive Ras GTP.
6. Add up to 200 µL of your sample to each well for a total amount of 10-100 µg of cell lysate. (see guide on IV. sample preparation.)
7. Incubate wells (sample lysate, positive control, and negative control) at room temperature (RT) with mild agitation for 1 hour.
8. Perform wash procedure as described in step 4.
9. Add 50 µL of prepared Primary Antibody Solution. Incubate at RT with mild agitation for 1 hour.
10. Perform wash procedure as described in step 4.
11. Add 50 µL of prepared Secondary Antibody Solution. Incubate at RT with mild agitation for 1 hour. Take out Chemiluminescent substrates and allow sitting out at RT for about an hour prior to use.
12. Perform wash procedure as described in step 4.
13. Final TBS rinse. Rinse each well with 200 µL of TBS, to remove Tween® 20. Repeat once.
14. Prepare the Chemiluminescent substrate by mixing the room temperature solution in a 2:1 reaction buffer to detection reagent ratio. Use substrate within 1-5 minutes of mixing.
15. Add 50 µL of the Chemiluminescent substrate to each well. Read results using a luminometer between 5 - 60 minutes after adding substrate. Typical expected results can be seen below. Note: results may very due to available luminometer settings and output measurements.

IV. Optional Procedure for Producing Positive/Negative Controls: GTPγS/GDP Treatment

Note: Samples that will not be loaded with GTPγS/GDP may be kept on ice during the loading of controls.

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 a second tube (negative control).
5. Incubate the tubes for 30 minutes at 30°C with agitation.
6. Dilute to desired concentration of cell lysate using Blocking/Binding Buffer and apply to well.

IX. CALCULATION OF RESULTS

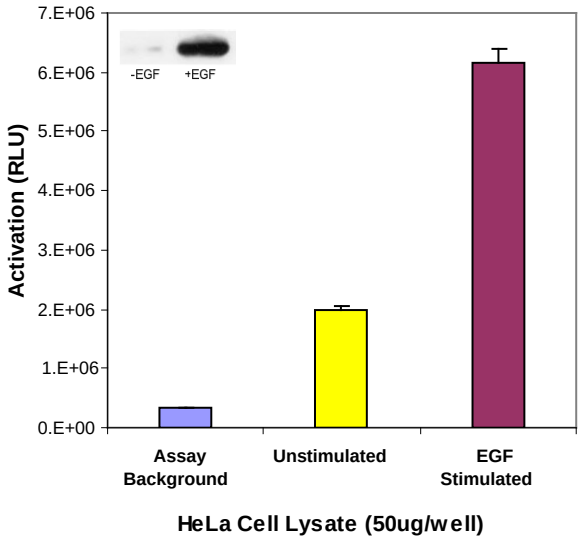


Figure 1. Ras Activation ELISA Assay Kit Performance

Following the protocol outlined in the manual, 50 µg of EGF-stimulated HeLa cell lysate (50 ng/mL EGF for 1 minute) shows elevated levels of activated Ras compared with the basal levels of activated Ras in 50 µg of unstimulated HeLa cell lysate. Assay background, which contains no cell lysate, is included to show the signal over noise ratio.

Insert: Western blot of Ras pull-down using 10µg Raf1 (14-278). Lysate was prepared from HeLa cells serum starved overnight, plus or minus 1 minute stimulation with 50 ng/mL EGF.

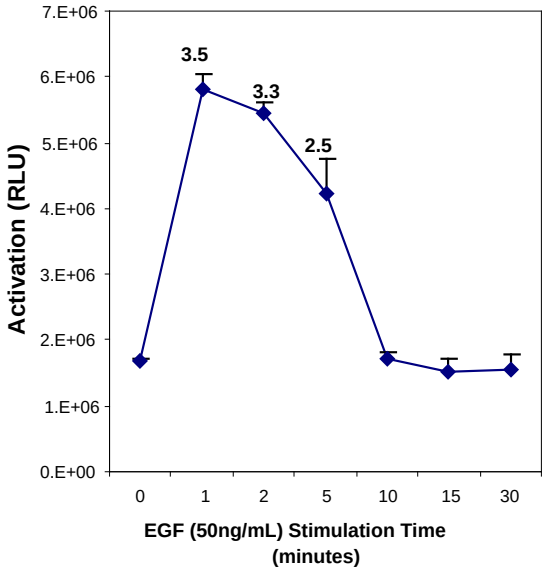


Figure 2. Time response of EGF stimulation in HeLa cells.

Lysate was prepared from HeLa cells stimulated with EGF for the time points indicated and assayed for Ras activity using the Ras Activation ELISA Assay Kit. The number indicated the fold excess over non-stimulated HeLa cells. Note: RLU numbers can vary between reading instrumentation and reading times post substrate addition.

X. Troubleshooting Guide

Problem	Potential Cause	Experimental Suggestions
No signal or weak signal in all wells	Missing components or key steps	Check to make sure all components were added in the appropriate steps and amounts.
	Secondary antibody conjugate or substrate is no longer active or has reduced activity	Test directly by adding HRP-conjugated secondary antibody with substrate.
	Plate reader or CCD camera settings are not optimal	Verify the measurement, read time, and filter on the luminometer.
	Incorrect storage temperatures	Items are to be stored at the appropriate storage temperatures. Performance can be negatively affected if reagents are not stored and used in the appropriate time period.
	Incorrect assay temperature Substrate preparation	Chemiluminescent Substrate needs to be warmed to room temperature prior to use, and mixed together 1 min prior to addition to well, and used within 1-5 minutes of mixing. Substrate needs to be mixed using the appropriate 2:1 ratio.
High background negative control wells	Inappropriate concentration of primary antibody.	Use concentration suggested of 1:10,000, dilution. Note the antibody is very sensitive; excess primary antibody will increase background.
	High measurement time.	Adjust camera/luminometer read settings.
	Inadequate washing	Make sure to follow the recommended protocol for washing. Ensure all wells are filled with wash buffer.
	Inadequate Blocking of negative control well.	Make sure to block well with the Block/Bind solution. Addition of greater than 50 µL of Blocking/Binding Solution per negative control well can be desirable.

Overly High signal in sample wells (prevents accurate reading on a luminometer)	Too much cell extract	Try using less cell extract
	Antibody titration is off	Try titering the primary and secondary antibodies with increased dilutions
	Washing	Wash wells thoroughly as described in protocol.
	Measurement time.	Adjust camera/luminometer read settings.
	Reading results too quickly	Allow reaction to sit up to an hour following addition of substrate, before reading if necessary.
No signal or weak signal in sample wells	Not enough extract per well	Increase amount of extract, not to exceed 500 µg/well or 200 uL volume.
	Ras is poorly activated or inactivated	Perform a time course in Ras activation in the studied cell line.
	Extracts are not from the correct species	The primary mouse monoclonal Ras antibody has specificity against H-, K-, and N-Ras, and is routinely evaluated by immunoblot on RIPA lysate from human A431 carcinoma cells, human HFF, Jurkat, Raji, mouse 3T3, CTLL, rat L-6 or PC-12 cell lysates

XI. REFERENCES

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2. Herrmann, C., Horn, G., Spaargaren, M., Wittinghofer, A., (1996) *J. Biol. Chem.* 271, 6794-6800
3. Adjei AA. (2001) *J. National Cancer Institute* 93 (14): 1062-1074
4. Downward, J. (2006) *Science* 314, 433-434

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