ab134640 –
Ras GTPase ELISA Kit

Instructions for Use

For the quantitative measurement of Human and rodent Ras GTPase activation in Human and rodent cell and tissue extracts.

This product is for research use only and is not intended for diagnostic use.

Version 4c Last Updated 15 June 2020

Table of Contents

1. BACKGROUND

Abcam's Ras GTPase ELISA kit is an *in vitro* ELISA (Enzyme-Linked Immunosorbent Assay) designed for accurate quantitative measurement of Human and rodent Ras GTPase activation in cell and nuclear extracts.

Abcam's Ras GTPase ELISA Kit is designed specifically for the study of Ras activation and can be used to study novel signaling pathways for activating Ras. Ras GTPase ELISA Kits contain a Raf-RBD protein fused to GST that will be coated onto the provided 96-well, glutathione-coated plate. The activated Ras contained in cellular extract specifically binds to Raf-RBD, while inactive Ras does not bind. Bound Ras is detected by incubating with a primary antibody that detects H-Ras in Mouse and H- & K-Ras in Human samples. Addition of a secondary antibody conjugated to horseradish peroxidase (HRP) and developing solution provides a sensitive chemiluminescent readout that is easily quantified by luminescence.

Small GTP-binding proteins (GTPases) are important regulators of signal transduction pathways. The small GTPase Ras acts as a key regulator of cellular functions including proliferation and differentiation and is also implicated in tumorigenesis, tumor invasion and morphogenesis. Oncogenic mutations in the Ras gene are present in approximately 30% of all Human cancers. Because of the critical role of Ras in tumor development, it is important to be able to screen novel signaling pathways for activating Ras. Traditional methods for monitoring Ras activation, such as Western blotting, are tedious and time consuming and not suitable to high-throughput analysis.

GTPases (also called GTP-binding proteins) are a family of enzymes that bind to and hydrolyze GTP, allowing them to function as molecular switches. When bound to GDP, the GTPase protein is in its inactive form. Activation is controlled by regulatory proteins called guanine nucleotide exchange factors (GEFs), which induce the release of GDP.

Because GTP is present in the cell in a large excess over GDP, the resulting empty nucleotide-binding site is filled by GTP and the GTPase is activated. Another class of proteins, GTPase-activating proteins (GAPs), speed up hydrolysis of GTP to GDP, inactivating the GTPase. The figure 1 below illustrates Ras activation.

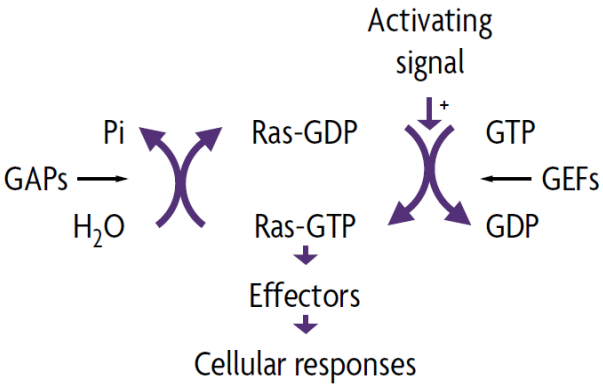


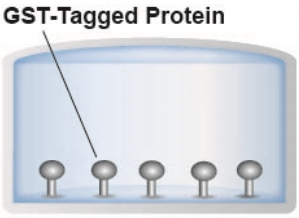
Figure 1. Ras Activation

The small GTPase Ras family regulates a variety of cell functions including proliferation and differentiation. Family members include Ras (H, K, N, R, M and TC21), Rap (1A, 1B, 2A and 2B) and Ral (A and B), and are characterized by similarities in their effector domains. Ras proteins consist of about 190 amino acid residues that are highly conserved in the N and C termini. Most variations between proteins occur near the C-terminal hypervariable domain, and this variation is presumed to be responsible for differences in function.

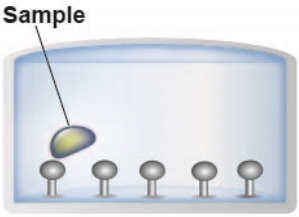
Activated Ras in turn activates several distinct effectors, such as the serine-threonine kinase Raf1, phosphoinositide 3´-kinase (PI3K) and RalGDS. One of the best characterized effector molecules activated by Ras is Raf kinase. Activation of Raf initiates a phosphorylation cascade involving MEK and ERK protein kinases leading to the activation of transcription factors like Elk.

Normally, Ras-signaling cascades are only transiently activated because GTPase's intrinsic hydrolyzing activity gradually converts GTP to GDP. This conversion is also enhanced by the presence of GAP proteins. However, there are mutant oncogenic Ras proteins that remain constitutively in the active GTP-bound form. Identified mutations are limited to a small number of sites that abolish GAP-induced hydrolysis of GTP, resulting in continuous stimulation of cellular proliferation. Oncogenic mutations in the ras gene are present in approximately 30% of all Human cancers. Colon and pancreatic cancers have mutations in the K-ras gene, urinary tract and bladder cancers have mutations in the H-ras gene, and mutations in N-ras are associated with leukemia.

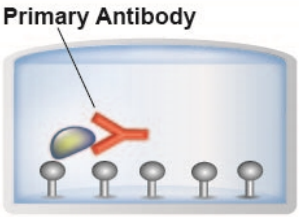
2. ASSAY SUMMARY



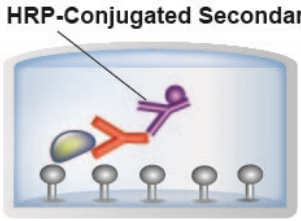
Prepare all reagents and samples as instructed. Plate is supplied pre-coated with glutathione. Coat plate with Raf-RBD protein (fused to GST). Incubate at 4°C.



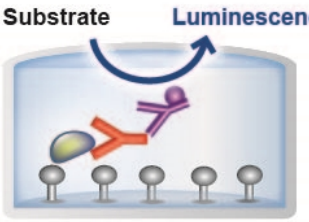
Wash each well. Add sample to appropriate wells. Activated Ras binds Raf-RBD, inactive Ras does not. Incubate at room temperature.



Wash each well. Add primary detection antibody. Incubate at room temperature.



Wash each well. Add HRP conjugated secondary antibody, which binds the primary antibody. Incubate at room temperature.



Wash each well. Add Chemiluminescent reagent. Read using a luminometer.

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Kit components arrive on dry ice. Immediately upon receipt, kit components must be stored at the temperatures indicated in the table.

Observe the storage conditions for individual prepared components in section 9. Reagent Preparation.

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
H-Ras antibody	1 x 11 µL	-20°C
Anti-rat HRP-conjugated IgG	1 x 11 µL (0.25 µg/µL)	-20°C
HeLa whole-cell extract (EGF treated)	2 x 40 µL (2.5 µg/µL)	-80°C
GST-Raf-RBD	4 x 25 µL (2 µg/µL)	-80°C
Protease Inhibitor Cocktail	1 x 500 µL	-20°C
Lysis/Binding Buffer	1 x 50 mL	+2-8°C
10X Wash Buffer	2 x 22 mL	+2-8°C
10X Antibody Binding Buffer	1 x 2.2 mL	+2-8°C
Chemiluminescent Reagent	1 x 2 mL	+2-8°C
Reaction Buffer	1 x 4 mL	+2-8°C
96-well assay plate	1 x unit	+2-8°C
Plate sealer	1 x unit	Room temperature

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Multi-channel pipettor
- Multi-channel pipettor reservoirs
- Rocking platform at room temperature and 4°C
- Microplate luminometer or CCD camera-coupled imaging system

These materials are not included in the kit, but will be required for the optional preparation of the Whole-Cell Extract (for protocol see section 11. Sample Collection and Storage):

- **Phosphate Buffered Saline (PBS)**

10X PBS	For 250 mL, mix:
0.1 M phosphate buffer, pH 7.5	3.55 g Na_2HPO_4 + 0.61 g KH_2PO_4
1.5 M NaCl	21.9 g
27 mM KCl	0.5 g

- Adjust to 250 mL with distilled water. Prepare a 1X PBS solution by adding 10 mL 10X PBS to 90 mL distilled water. Sterilize the 1X PBS by filtering through a 0.2 µm filter. The 1X PBS is at pH 7.5. Store the filter-sterilized 1X PBS solution at 4°C.

These materials are not included in the kit, but will be required for the optional preparation of Positive and Negative controls (for suggested protocol see section 11. Sample Collection and Storage):

- 10 mM GTPγS
- 100 mM GDP
- 0.5M EDTA pH 8.0
- 1M $MgCl_2$

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

8. TECHNICAL HINTS

- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Complete removal of all solutions and buffers during wash steps.
- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**

9. REAGENT PREPARATION

Equilibrate all reagents and samples to room temperature (18-25°C) prior to use.

9.1 Complete Lysis/Binding Buffer

We provide an excess of Lysis/Binding Buffer in order to perform the assay AND to prepare customized cell extracts. Prepare the amount of Complete Lysis/Binding Buffer required for the assay by adding 10 µL of Protease Inhibitor Cocktail per mL of Lysis/Binding Buffer.

Some of the protease inhibitors lose their activity after 24 hours once diluted. Therefore, we recommend using the Complete Lysis/Binding Buffer immediately for cell lysis. The remaining amount should be discarded if not used in the same day.

9.2 1X Wash Buffer

Prepare the amount of 1X Wash Buffer required for the assay as follows: For every 10 mL of 1X Wash Buffer required, dilute 1 mL 10X Wash Buffer with 9 mL distilled water. Mix gently to avoid foaming.

The 1X Wash Buffer may be stored at 4°C for one week. The Tween 20 contained in the 10X Wash Buffer may form clumps, therefore homogenize the buffer by vortexing for 2 minutes prior to use.

9.3 1X Antibody Binding Buffer

Prepare the amount of 1X Antibody Binding Buffer required for the assay as follows: For every 10 mL of 1X Antibody Binding Buffer required, dilute 1 mL 10X Antibody Binding Buffer with 9 mL distilled water. Mix gently to avoid foaming.

Discard remaining 1X Antibody Binding Buffer after use. The BSA contained in the 10X Antibody Binding Buffer may form clumps, therefore homogenize the buffer by warming to room temperature and vortexing for 1 minute prior to use.

9.4 **Diluted Primary Antibody**

The primary Ras antibody recognizes H- and K-Ras in Human and H-Ras in rodent samples. The supplied antibody will be diluted 1:500 in 1X Antibody Binding Buffer.

Avoid multiple freeze/thaw cycles.

9.5 **Diluted HRP-conjugated Secondary Antibody**

HRP-conjugated anti-rat IgG is used as the secondary antibody to detect bound primary antibody. The supplied antibody will be diluted 1:5000 in 1X Antibody Binding Buffer. This dilution should be made by performing a 1:10 dilution followed by a 1:500 dilution.

Avoid multiple freeze/thaw cycles.

9.6 **Chemiluminescent Working Solution**

The Chemiluminescent Reagent and Reaction Buffer should be warmed to room temperature before use. These components are light sensitive, therefore, we recommend avoiding direct exposure to intense light during storage. Prior to use, place the Chemiluminescent Reagent and Reaction Buffer at room temperature for at least 1 hour. In a separate container, mix 1 volume of Chemiluminescent Reagent with 2 volumes of Reaction Buffer to prepare the Chemiluminescent Working Solution.

The Chemiluminescent Working Solution is stable for several hours. After the Chemiluminescent Working Solution is aliquoted into the wells, discard the remaining solution.

9.7 **GST-Raf-RBD**

The GST-Raf-RBD contains a Ras Binding Domain and is used to capture activated Ras on the glutathione-coated plate.

GST-Raf-RBD must be aliquoted into small fractions to avoid freeze/thaws. Four tubes of the GST-Raf-RBD are provided and must be stored at -80°C upon receipt.

10. CONTROL PREPARATION

Positive Control (HeLa whole cell extract)

The HeLa whole-cell extract (EGF treated) is provided as a positive control for Ras activation. Sufficient extract is supplied for 8 reactions per plate. This extract is optimized to give a strong signal when used at 25 µg/well.

We recommend aliquoting the extract in 21 µL fractions and storing at -80°C. Avoid multiple freeze/thaw cycles of the extract.

Note: The HeLa whole-cell extract (EGF treated) is sensitive to GTP hydrolysis at 4°C, thus we recommend thawing it no more than 15 minutes prior to use.

11. SAMPLE COLLECTION AND STORAGE

Preparation of Whole-Cell Extract

- **For reagent preparation for this protocol see section 6. Materials Required, not Supplied. Exception: for preparation of Complete Lysis/Binding Buffer see section 9. Reagent Preparation.**
- **This procedure can be used for a confluent cell layer of 10 cm^2 (100 mm dish) or 2 x 10^7 cells.**

11.1.1 Treat the cells as required for Ras activation.

11.1.2 Wash the cells with 5 mL ice-cold PBS (10 mM phosphate buffer, pH 7.5, 150 mM NaCl).

11.1.3 For adherent cells add 500 µL of Complete Lysis/Binding Buffer and scrape cells For suspension cells resuspend cell pellet in 1 mL Complete Lysis/Binding Buffer.

11.1.4 Transfer cells to a microcentrifuge tube. Incubate 15 minutes at 4°C.

11.1.5 Vortex tube for 10 seconds and then centrifuge for 10 minutes at 14,000 rpm at 4°C.

11.1.6 Collect the supernatant at 4°C.

11.1.7 Measure the protein content by a Bradford-based assay.

11.1.8 For best results, extracts should be used immediately in the Ras GTPase ELISA.

Optional – GTPγS or GDP Treatment

- **The protocol below is provided as an optional procedure for the production of positive and negative controls for Ras activation. GTPγS acts as an activator while GDP acts as an inhibitor to Ras activation. Use > 200 µg of extract for each treatment.**

11.2.1. Dilute the test extracts to desired concentration in Complete Lysis/Binding Buffer (>200 µg per well is recommended).

11.2.2. To each tube, add 0.5M EDTA pH 8.0 to a final concentration of 10 mM.

11.2.3. To each tube, add 10 mM GTPγS or 100 mM GDP to a final concentration of 0.1 mM and 1.0 mM, respectively.

11.2.4. Incubate at 30°C for 15 minutes.

11.2.5. To each tube, add 1M $MgCl_2$ to a final concentration of 60 mM.

11.2.6. Extracts should be used immediately in the Ras ELISA.

12. PLATE PREPARATION

- The 96 well plate included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
- For each assay performed, a minimum of 2 wells must be used as blanks, omitting primary antibody from well additions.
- For statistical reasons, we recommend each sample, control and blank should be assayed with a minimum of two replicates (duplicates).
- Well effects have not been observed with this assay.
- If less than 8 wells in a strip are required, cover the unused wells with a portion of the plate sealer while you perform the assay.
- The content of these wells is stable at room temperature if kept dry and, therefore, can be used later for a separate assay. Store the unused plates in the aluminium pouch at 4°C.
- Use the strip holder for the assay.

13. ASSAY PROCEDURE

- **Equilibrate all materials and prepared reagents to room temperature prior to use.**
- **It is recommended to assay all standards, controls and samples in duplicate.**
- **Prepare all reagents, controls, and samples as directed in the previous sections.**
- **IMPORTANT: For optimal kit performance, kit components must be stored at the recommended storage temperatures indicated in section 5. Materials Supplied for 24 hours prior to use.**

Binding of Ras

13.1 Add 2 µg of GST-Raf-RBD diluted in 50 µL of Complete Lysis/Binding Buffer to each well to be used. (1 µL of GST-Raf-RBD in 49 µL Complete Lysis/Binding Buffer per well).

13.2 Use the provided adhesive cover to seal the plate. Incubate for 1 hour at 4°C with mild agitation (100 rpm on a rocking platform).

13.3 Wash each well 3 times with 200 µL 1X Wash Buffer. For each wash, flick the plate over a sink to empty the wells, then tap the inverted plate 3 times on absorbent paper towels.

13.4 Sample wells: Dilute test extracts to desired concentration in Complete Lysis/Binding Buffer. Sample can be used at 50-200 µL per well, depending on stock concentration. We recommend using 10-100 µg of extract diluted in Complete Lysis/Binding Buffer per well.

Positive control wells: Thaw the provided HeLa (EGF treated) extract on ice for no more than 15 minutes prior to use. Add 25 µg of this extract diluted in 50 µL of Complete Lysis/Binding Buffer per well (10 µL of extract in 40 µL of Complete Lysis/Binding Buffer per well).

Blank wells: Add 50 µL Complete Lysis/Binding Buffer only per well.

13.5 Cover the plate and incubate for 1 hour at room temperature with mild agitation (100 rpm on a rocking platform).

13.6 Wash each well 3 times with 200 µL 1X Wash Buffer (as described in Step 13.3).

Binding of primary antibody

13.7 Add 50 µL diluted H-Ras antibody (1:500 dilution in 1X Antibody Binding Buffer) to wells.

13.8 Cover the plate and incubate for 1 hour at room temperature without agitation.

13.9 Wash the wells 3 times with 200 µL 1X Wash Buffer (as described in Step 13.3).

Binding of secondary antibody

13.10 Add 50 µL diluted HRP antibody (1:5,000 dilution in 1X Antibody Binding Buffer) to all wells being used.

13.11 Cover the plate and incubate for 1 hour at room temperature without agitation.

13.12 During this incubation, place Chemiluminescent Reagent and Reaction Buffer at room temperature.

13.13 Wash the wells 4 times with 200 µL 1X Wash Buffer (as described in Step 13.3).

Chemiluminescent detection

13.14 Add 50 µL room-temperature Chemiluminescent Working Solution to all wells being used.

13.15 Read chemiluminescence using a luminometer or CCD camera system. Readings should be taken within 15 minutes to minimize changes in signal intensity.

14. TYPICAL DATA

This data is provided for demonstration purposes only.

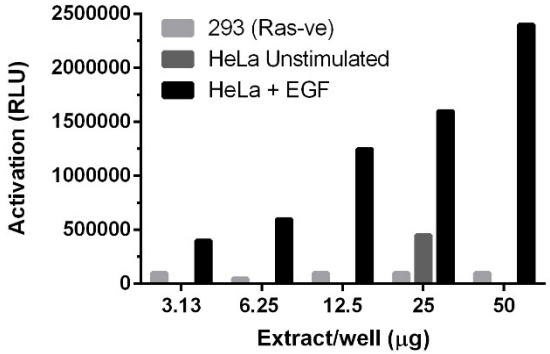


Figure 2. Quantification of activated Ras: Increasing amounts of whole-cell extracts from unstimulated 293T/17 and EGF stimulated HeLa cells were assayed for Ras activity using the Ras GTPase ELISA Kit. To illustrate the Kit's specificity for activated Ras, 293T/17 cells which do not contain basal levels of activated Ras were used as a negative control. Data was also shown for unstimulated HeLa cells, which do contain basal levels of activated Ras.

15. ASSAY SENSITIVITY

Detection limit: > 3 µg whole-cell extract/well or > 0.6 ng purified protein/well.

Range of Detection: The Ras GTPase ELISA Kit provides quantitative results from 3 to 25 µg of cell extract/well.

16. ASSAY SPECIFICITY

Cross-reactivity: Ras GTPase ELISA Kit specifically detects activated H- and K-Ras in Human and H-Ras in rodent samples.

17. TROUBLESHOOTING

Problem	Cause	Solution
No signal or weak signal in all wells	Omission of key reagent	Check that all reagents have been added in the correct order
	Substrate or conjugate is no longer active	Test conjugate and substrate for activity
	Enzyme inhibitor present	Sodium azide will inhibit the peroxidise reaction, follow our recommendations to prepare buffers
	Plate reader or CCD camera settings not optimal	Verify the measurement mode and filter settings in the plate reader or CCD camera
	Incorrect storage temperature	Kit components arrive on dry ice. Upon receipt, kit contents should be stored at recommended temperatures listed on page 5 of the manual for at least 24 hours before use. Studies have indicated that kit performance may be negatively impacted if reagents are stored incorrectly or used upon receipt.
No signal or weak signal in all wells	Incorrect assay temperature	Bring substrate to room temperature before use
	Inadequate volume of Chemiluminescent Working Solution	Check to make sure that correct volume is delivered by pipette

Problem	Cause	Solution
High background in all wells	Measurement time too long	Reduce integration time or exposure time on luminometer or CCD camera
	Concentration of antibodies too high	Increase antibody dilutions
	Inadequate washing	Ensure all wells are filled with Wash Buffer and follow washing recommendations
High background in sample wells	Too much sample per well	Decrease amount of sample
	Concentration of antibodies too high	Perform antibody titration to determine optimal working concentration. Start using 1:1,000 for primary antibody and 1:10,000 for secondary antibody. The sensitivity of the assay will be decreased
No signal or weak signal in sample wells	Not enough extract per well	Increase amount of extract not to exceed 500 µg/well
	Ras is poorly expressed or inactivated in samples	Perform a time course for Ras activation in the studied sample
	Samples are not from correct origin	Refer to cross-reactivity information

18. NOTES

UK, EU and ROW
Email: technical@abcam.com | Tel: +44-(0)1223-696000

Austria
Email: wissenschaftlicherdienst@abcam.com | Tel: 019-288-259

France
Email: supportscientifique@abcam.com | Tel: 01-46-94-62-96

Germany
Email: wissenschaftlicherdienst@abcam.com | Tel: 030-896-779-154

Spain
Email: soportecientifico@abcam.com | Tel: 911-146-554

Switzerland
Email: technical@abcam.com
Tel (Deutsch): 0435-016-424 | Tel (Français): 0615-000-530

US and Latin America
Email: us.technical@abcam.com | Tel: 888-77-ABCAM (22226)

Canada
Email: ca.technical@abcam.com | Tel: 877-749-8807

China and Asia Pacific
Email: hk.technical@abcam.com | Tel: 400 921 0189 / +86 21 2070 0500

Japan
Email: technical@abcam.co.jp | Tel: +81-(0)3-6231-0940

www.abcam.com | www.abcam.cn | www.abcam.co.jp