ACTIVE MOTIF®

Enabling Epigenetics Research

NRAS In-well Lysis ELISA Kit

Catalog No. 52099

(Version A1)

Contents

Overview

Small GTPase RAS proteins bind to and hydrolyze GTP, allowing them to function as molecular switches, cycling between an active (GTP-bound) and inactive (GDP-bound) state. This switch regulates signal transduction pathways involved in cellular functions including proliferation, differentiation, and apoptosis. RAS proteins exist as three major isoforms: HRAS, KRAS, and NRAS, each containing about 190 amino acids that share 80-90% sequence identity.

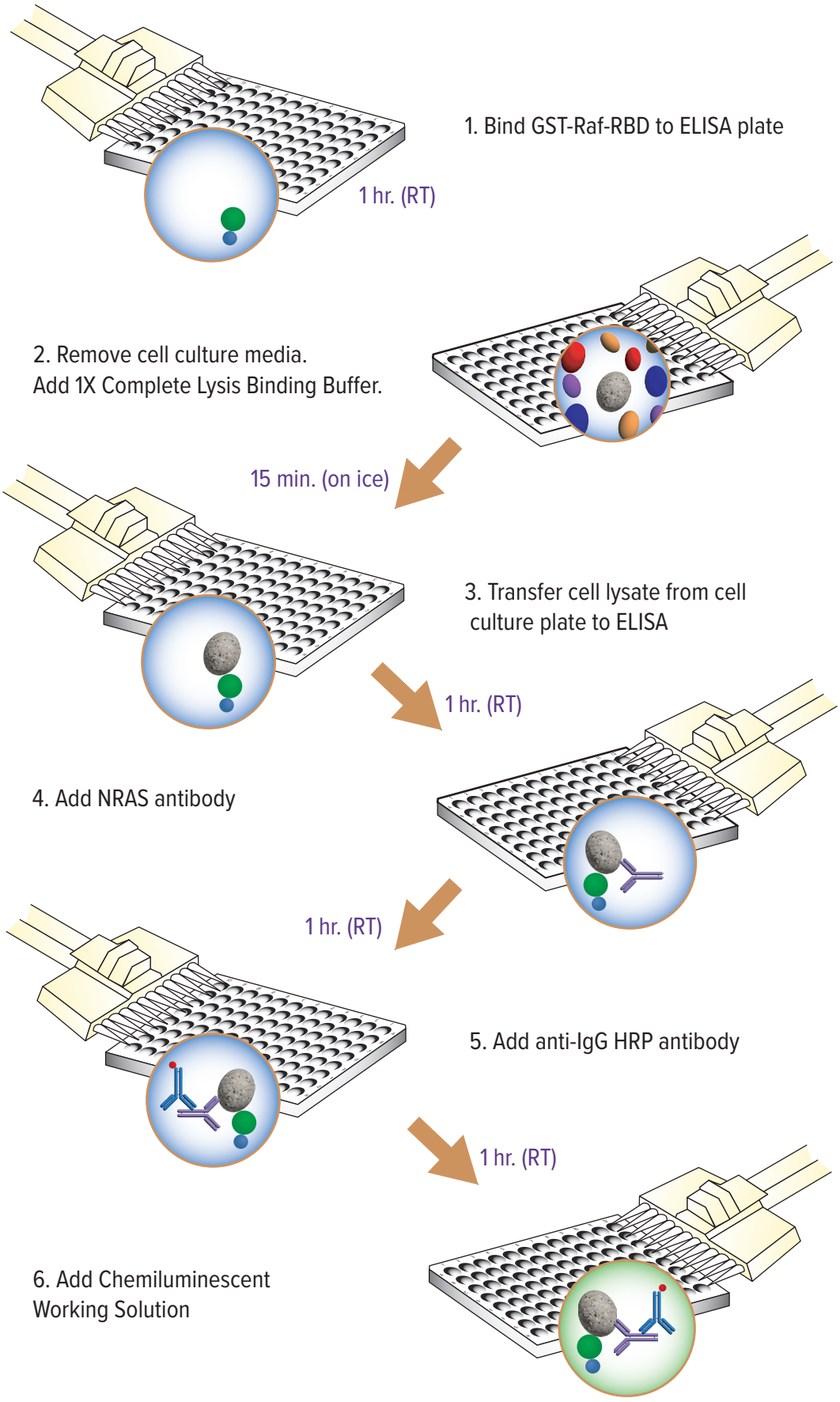
RAS is one of the most frequently mutated genes in cancer. Gain-of-function missense mutations which promote oncogenesis cluster at codons 12, 13, and 61 of KRAS, NRAS and HRAS resulting in enhanced GTP binding due to fast exchange of nucleotide and/or impairment of GAP (GTPase Activating Protein) binding. Although RAS mutations are all activating, they vary in their oncogenic potential and frequency in different tissues, with NRAS mutations being the second most prevalent.

The NRAS In-Well ELISA Kit, which measures active human NRAS via chemiluminescent detection, is an extension of the existing RAS Kits. The NRAS In-Well ELISA Kit is designed specifically for the study of NRAS activation and inhibition. The assay allows for direct in-well lysis of cells cultured in 96-well plate format, either directly in media or following removal of media. The NRAS In-well ELISA Kit contains a Raf-RBD protein fused to GST that will be coated onto the provided 96-well, glutathione-coated plate. Active RAS contained in the cellular extract binds to Raf-RBD, while inactive RAS does not and is washed away. Bound NRAS is then detected by incubation with a primary antibody that specifically detects NRAS. Addition of a secondary antibody conjugated to horseradish peroxidase (HRP) and developing solution provides a sensitive chemiluminescent readout that is easily quantified by luminescence. The 96-well plate is suitable for manual use or high-throughput screening.

The Positive Control Lysate (SK-MEL-2), 2.5 µg/µL is provided as a positive control for NRAS activation and is optimized to give a strong signal with an assay window (signal/background) of more than 10X when used at 40 µL/well.

Product	Format	Catalog No.
NRAS In-well Lysis ELISA Kit	1 x 96 rxns	52099

Flow Chart of Process



Kit Performance and Benefits

The NRAS In-well ELISA Kit is for research use only. Not for use in diagnostic procedures.

Recommended cell seeding density: The optimal cell seeding density for a 96-well culture plate for use in the ELISA will depend on both the cell line and incubation time. However, some suggested ranges are given below.

SK-MEL-2: 20×10^5 - 5×10^5 cell seeding density

Range of Detection: The NRAS In-well Lysis ELISA kit will give relative quantitation of NRAS in the range of 20 µg - 50 µg whole cell extract/well.

Cross-reactivity: The NRAS In-well Lysis ELISA kit is human NRAS specific. It does not detect the human HRAS or NRAS isoforms. Predicted cross reactivity with mouse NRAS.

Positive Control: The Positive Control Lysate (SK-MEL-2) is provided as a positive control for NRAS activation at a concentration of 2.5 µg/µL. Each vial contains enough material for 4 reactions per plate. The extract is optimized to give a strong signal with an assay window (signal/background) of more than 30X when used at 50 µL/well.

Assay time: 4.5 hours.

Kit Components and Storage

Please store each component at the temperature indicated in the table below upon receipt of the kit. Kit components must be stored at the temperatures listed below for 24 hours prior to use.

Reagents	Quantity	Storage
GST-Raf-RBD	100 µL	-80°C
Positive Control Lysate (SK-MEL-2)	40 µL	-80°C
Recombinant NRAS Antibody	20 µL	-80°C
HRP-conjugated Secondary Antibody	20 µL	-20°C
Protease Inhibitor Cocktail (PIC)	2 x 101 µL	-20°C
5X Lysis Binding Buffer	4 mL	4°C
10X Wash Buffer AM2	29 mL	4°C
1X Antibody Binding Buffer	14 mL	4°C
Chemiluminescent Reagent	1.9 mL	4°C
Reaction Buffer	3.8 mL	4°C
96-well assay plate	1 ea	4°C
Plate sealer	4 ea	4°C

Additional Materials Required

- 96-well microplates
- 5 or 15-mL Falcon™ tubes
- 2-200 µL multi-channel pipettes
- Reagent Reservoirs
- Pipettes
- Pipette tips
- 1.5 mL microtubes
- Microplate shaker
- Vortex
- Rocking platform at room temperature (22°C) and 4°C
- Distilled water
- HandyStep Repeating Pipette with syringe tips (optional)
- Automated plate washer (optional)
- 1X PBS (optional for washing cells) pH 7.4-7.6
- Microplate luminometer or CCD camera-coupled imaging system

Protocol

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING

For optimal kit performance, kit components must be stored at the recommended storage temperatures indicated on page 4 of the manual 24 hours prior to use. A multi-channel pipette or repeating pipette and reagent reservoirs may be used for dispensing all reagents.

Prepare Buffers

Before beginning, prepare 1X Complete Lysis Binding Buffers and 1X Wash Buffer.

1X Complete Lysis Binding Buffer (for cell lysis)

Preparation of 1X Complete Lysis Binding Buffer is required for preparing whole cell lysate for the ELISA. For lysis of cells in a 96-well culture plate, prepare a total of 15 mL 1X Complete Lysis Binding Buffer by combining 3.0 mL 5X Lysis Binding Buffer, 11.85 mL distilled water, and 150 µL Protease Inhibitor. Place on ice. Protease inhibitors lose their activity after 24 hours once diluted, so make the 1X Complete Lysis Binding Buffer the day of use. The remaining amount should be discarded if not used on the same day.

Alternatively, whole cell extracts can be prepared and quantified separately as described in the Appendix, Section A. Preparation of Whole-Cell Extract, diluted in 1X Complete Lysis Binding Buffer and transferred to the ELISA.

1X Complete Lysis Binding Buffer (for binding GST-Raf-RBD to ELISA plate)

Preparation of 1X Complete Lysis Binding Buffer is required for binding the GST-Raf-RBD protein to the ELISA Plate. Per 96-well ELISA, prepare 5.0 mL 1X Complete Lysis Binding Buffer by combining 1.0 mL 5X Lysis Binding Buffer, 3.95 mL distilled water, and 15 µL Protease Inhibitor. Place on ice. Protease inhibitors lose their activity after 24 hours once diluted, so make the 1X Complete Lysis Binding Buffer the day of use. The remaining amount should be discarded if not used on the same day.

1X Wash Buffer

Per 96-well ELISA prepare 300 mL of 1X Wash Buffer by mixing 29 mL 10X Wash Buffer with 261 mL distilled water. Mix gently to avoid foaming. The Tween 20 contained in 10X Wash Buffer AM2 may form clumps. If this occurs, homogenize the buffer by mixing gently. Place at room temperature (22°C) before use. The 1X Wash Buffer may be stored at 4°C for one week.

Step 1: Treatment of Cells

The number of seeded cells and incubation period should be optimized for each cell line. For SK-MEL-2 cells a recommended seeding range is 20 x 10^5 - 5 x 10^5 cells per well in a 96-well culture plate. We suggest 3-4 replicates per experimental condition. Treat the cells with 100 µL of media containing NRAS activating or inhibiting components as required.

For best results, the GST-Raf-RBD should be freshly bound to the ELISA plate immediately before cell lysis. We suggest doing Step 2: Binding the GST-Raf-RBD to Plate, 1 hour before NRAS activation/inhibition will be complete. Then proceed with Step 3: In-well Cell Lysis.

Note that once the media is removed from the cells, they can be stored at -80°C and then lysed on the day of the day of ELISA. Cell lysates can also be stored frozen at -80°C before transferring to the ELISA. However, a reduction in signal may be observed.

Step 2: Binding GST-Raf-RBD to Plate

Thaw GST-Raf-RBD on ice immediately before use. Each vial of GST-Raf-RBD contains enough to coat one 96-well ELISA plate. If only a partial plate will be used, aliquot into small fractions to avoid freeze/thaws and store at -80°C.

1. For a 96-well ELISA prepare 5.0 mL GST-Raf-RBD Solution by combining 100 µL GST-Raf-RBD with 4.9 mL Complete Lysis Binding Buffer. Invert 10 times to mix. Discard any unused diluted GST-Raf-RBD.
2. Dispense 50 µL GST-Raf-RBD mixture into each well of the ELISA plate.
3. Use the provided adhesive cover to seal the plate. Incubate for 1 hour at room tempurature (22°C) without agitation.
4. Wash each well 3 times with 200 µL 1X Wash Buffer. For each wash, flick the plate over a waste container to empty the wells, then strongly tap the inverted plate more than 5 times on absorbent paper towels until there is no observable moisture on the paper towel.

Step 3: In-well Cell Lysis

5. Remove the media from the 96-well culture plate by inverting over a large reservoir. Then tap the inverted plate over a paper towel until there is no observable moisture on the paper towel. Add 100 µL 1X Complete Lysis Binding Buffer to each well of the culture plate using a multichannel pipettor.
6. Firmly seal the plate. Place the sealed plate on ice for 20 minutes. For some cell lines, lysis may be improved by placing the plate on a microplate shaker at 20-30 rpm and 4°C for 20 minutes.
7. Carefully remove the plate seal. Mix by pipetting up and down 8 times. For the assay, 50 µL of cell lysate will be transferred from each well of the culture plate to the ELISA plate.

Step 4: Add Samples to Wells

Sample wells: Transfer 50 µL of lysed whole cells from each culture well to the ELISA for the assay. Be sure the lysed cells are resuspended by pipetting up and down 8 times before the sample is transferred.

Positive control wells: Thaw the Positive Control Lysate (SK-MEL-2) on ice for no more than 15 minutes prior to use. Each vial contains enough material for 4 reactions per plate. Mix 15 µL of extract with 35 µL of 1X Complete Lysis Binding Buffer per well. Transfer 50 µL to each positive control well.

Blank wells: Add 50 µL 1X Complete Lysis Binding buffer to each well.

8. Cover the plate and incubate for 1 hour at room temperature (22°C) with mild agitation (Nutating rocker or 100 rpm on a rocking platform).
9. Wash each well 3 times with 200 µL 1X Wash Buffer. For each wash, flick the plate over a waste container to empty the wells, then tap the inverted plate 3 times on absorbent paper towels.

Step 5: Binding of primary antibody

10. Spin vial of supplied Recombinant NRAS Antibody briefly in a microcentrifuge before opening to collect contents to the vial bottom. The Recombinant NRAS Antibody will first be diluted 1:10 and then by 1:1,000* in 1X Antibody Binding Buffer according to the table below. Mix the 1:10 dilution with a pipette for 7-8 times. Vortex the 1:1000 dilution for about 5 seconds and invert 5 times to mix. If not using the entire vial of Recombinant NRAS antibody at one time we suggest aliquoting into smaller volumes to avoid multiple freeze and thaw cycles.

Reagents	Components	Volume
NRAS Antibody (1:10 dilution)	Recombinant NRAS Antibody	4.0 µL
	1X Antibody Binding Buffer	36.0 µL
	Total	**40.0 µL**
Diluted NRAS Antibody	NRAS Antibody (1:10 dilution)	6 µL*
	1X Antibody Binding Buffer	6.0 mL
	Total	**6.0 mL**

* See Troubleshooting Guide for guidance on increasing or decreasing signal or background.

11. Add 50 µL Diluted NRAS antibody to each well.
12. Cover the plate and incubate for 1 hour at room temperature (22°C) without agitation.
13. Wash each well 3 times with 200 µL 1X Wash Buffer. For each wash, flick the plate over a waste container to empty the wells, then tap the inverted plate 3 times on absorbent paper towels.

Step 6: Binding of Secondary Antibody

14. Spin the vial of supplied HRP-conjugated Secondary Antibody briefly in a microcentrifuge before opening to collect contents to the vial bottom. The HRP-conjugated Secondary Antibody will first be diluted 1:400 and then by 1:500* in 1X Antibody Binding Buffer according to the table below. Quick vortex and Invert 5 times to mix between dilutions. If not using the entire vial of HRP-conjugated Secondary Antibody at one time we suggest aliquoting into smaller volumes to avoid multiple freeze and thaw cycles.

Reagents	Components	Volume
Secondary Antibody (1:400 dilution)	HRP-conjugated Secondary Antibody	4.0 µL
	1X Ab Binding Buffer	1.6 mL
	Total	**1.6 mL**
Diluted Secondary Antibody	Secondary Antibody (1:400 dilution)	12 µL*
	1X Ab Binding Buffer	6 mL
	Total	**18 mL**

* See Troubleshooting Guide for guidance on increasing or decreasing signal or background.

15. Add 50 µL Diluted Secondary antibody per well.

16. Cover the plate and incubate for 1 hour at room temperature (22°C) without agitation.

17. During this incubation, place Chemiluminescent Reagent and Reaction Buffer at room temperature (22°C). These components are light sensitive. Avoid direct exposure to intense light.

18. Wash each well 4 times with 200 µL 1X Wash Buffer. For each wash, flick the plate over a waste container to empty the wells, then tap the inverted plate 3 times on absorbent paper towels.

Step 7: Chemiluminescent detection

Be sure the Chemiluminescent Reagent and Reaction Buffer was placed at room temperature (22°C) for at least 1 hour.

19. Per 96-well ELISA, prepare 5.7 mL of Chemiluminescent Working Solution by mixing 1.9 mL Chemiluminescent Reagent and 3.8 mL Reaction Buffer. Invert several times to mix. The Chemiluminescent Working Solution is stable for several hours. After use, discard any remaining solution.

20. Add 50 µL room-temperature Chemiluminescent Working Solution to all wells being used. Read chemiluminescence using a luminometer. Readings should be taken within 10 minutes to minimize changes in signal intensity. As the luminescent signal will can change over time, we have found that it's best to use a short signal integration interval (0.2 seconds) for each well so that all wells will read similarly. For experiments involving multiple plates, it is recommended to stagger the addition of the chemiluminescent solution so that each plate is measured within the same time frame.

Appendix

Section A. Preparation of Whole-Cell Extract

To prepare whole cell extracts for use in the ELISA, the following protocol can be used for a 100 mm culture dish or 2 x 10^7 cells. Additional 5X Lysis Binding Buffer and Protease Inhibitor Cocktail can be purchased as Cat No. 52110.

1. For each 100 mm culture dish or 2 x 10^7 cells, prepare 500 µL 1X Complete Lysis Binding Buffer by mixing 5 µL Protease Inhibitor Cocktail, 100 µL 5X Lysis Binding Buffer, and 395 µL distilled water.

Note: Protease inhibitors lose their activity after 24 hours once diluted, so make the 1X Complete Lysis Binding Buffer the day of use. The remaining amount should be discarded if not used the same day.

2. Remove culture media from cells.
3. For adherent cells add 500 µL of 1X Complete Lysis Binding Buffer to the plate and scrape cells with a rubber policeman. For pelleted cells resuspend cell pellet in 500 µL 1X Complete Lysis Binding Buffer.
4. Transfer suspended cells to a microcentrifuge tube. Incubate 15 minutes on ice.
5. Vortex tube for 10 seconds and then centrifuge for 10 minutes at 14,000 rpm at 4°C.
6. Collect the supernatant at 4°C.
7. Measure the protein content using a BCA Protein Assay such as the Thermo Scientific Pierce BCA Protein Assay Kit, Cat No. 23225.
8. Extracts can be aliquoted and frozen at -80°C for later use. However, for best results, use immediately in the ELISA. Dilute whole-cell extracts in 1X Complete Lysis Binding Buffer to desired concentration and transfer 50 µL to each ELISA well.

Troubleshooting Guide

Problem/Question	Possible cause	Recommendation
No signal or weak signal in all wells	Omission of key reagent	Check that all reagents have been added in all wells in the correct order.
	Substrate or conjugate is no longer active	Test conjugate and substrate for activity by mixing HRP and Chemiluminescent Working Solution together.
	Plate reader settings not optimal	Verify that measurement mode is set to luminescence.
	Incorrect assay temperature	Bring substrate to room temperature (22°C) before using.
	Inadequate volume of Chemiluminescent Working Solution	Check to make sure that correct volume is delivered by pipette.
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	Concentration of antibodies is too high	Increase antibody dilutions.
No signal or weak signal in sample wells	Not enough extract per well	Increase number of cells seeded in the culture plate.
	NRAS is poorly activated or inactive	Perform a time course for NRAS activation in the studied cell line.
	Extracts are not from correct species	Refer to cross-reactivity information.
	Concentration of antibodies is too low	Decrease antibody dilutions, for example by reducing the secondary dilutions from 1:1000 to 1:500.

Technical Services

If you need assistance at any time, please call or send an e-mail to Active Motif Technical Service at one of the locations listed below.

North America	Toll free:	877 222 9543
	Direct:	760 431 1263
	Fax:	760 431 1351
	E-mail:	tech_service@activemotif.com
Europe	Direct:	+32 (0)2 653 0001
	Fax:	+32 (0)2 653 0050
	E-mail:	eurotech@activemotif.com
Japan	Direct:	+81 (0)3 5225 3638
	Fax:	+81 (0)3 5261 8733
	E-mail:	japantech@activemotif.com
China	Direct:	(86)-21-20926090
	Cell Phone:	18521362870
	E-mail:	techchina@activemotif.com