KRAS In-well Lysis ELISA Kit

Catalog No. 52100 & 52105

(version A1)

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Overview

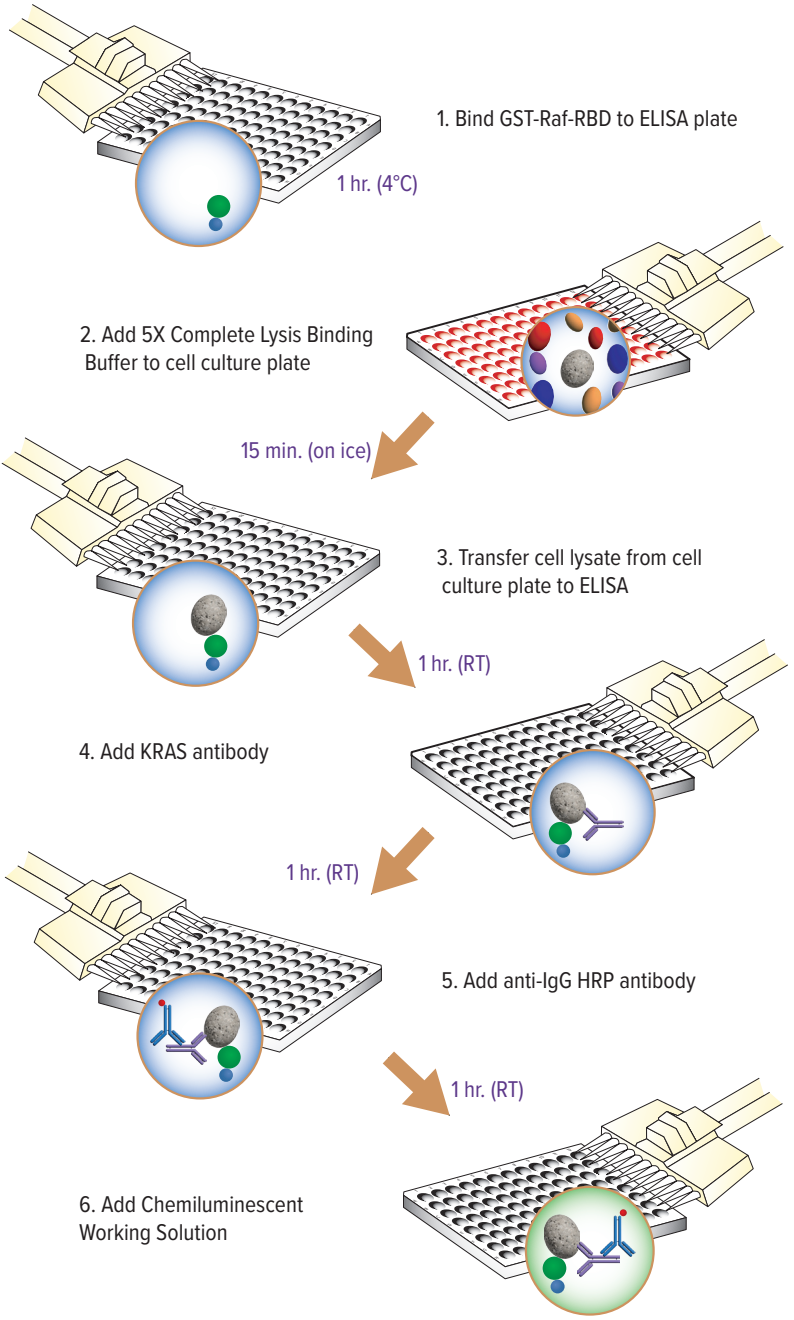
Small GTPase RAS proteins bind to and hydrolyze GTP, allowing them to function as molecular switches, cycling between an active (GTP-bound) and inactive (GDP-bound) state. This switch regulates signal transduction pathways involved in cellular functions including proliferation, differentiation, and apoptosis. RAS proteins exist as three major isoforms: HRAS, NRAS, and KRAS, 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 KRAS mutations being the most prevalent.

The KRAS In-Well ELISA Kit, which measures active human KRAS via chemiluminescent detection, is an extension of the existing RAS GTPase Chemi ELISA Kit (Cat no. 52097). The KRAS In-Well ELISA Kit is designed specifically for the study of KRAS activation and inhibition. The new assay allows for direct in-well lysis of cells cultured in 96-well plate format, either directly in media or following a PBS wash step. The KRAS 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 KRAS is then detected by incubation with a primary antibody that specifically detects KRAS. 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.

product	format	catalog no.
KRAS In-well Lysis ELISA Kit	1 x 96 rxns	52100
KRAS In-well Lysis ELISA Kit	5 x 96 rxns	52105

Flow Chart of Process



Kit Performance and Benefits

The KRAS 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. Guidelines are given below for two cell lines.

HeLa: 7.0 x 10^4 - 16 x 10^4 cell seeding density

Aspc1: 2.0 x 10^4 - 32 x 10^4 cell seeding density

Range of Detection: The KRAS In-well Lysis ELISA kit will give relative quantitation of KRAS in the range of 6.25 µg - 50 µg whole cell extract/well.

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

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.

Catalog no.	52100	52105	Storage
Reactions	96	5 x 96	
GST-Raf-RBD	110 µl	5 x 110 µl	-80°C
Positive Control Lysate (AsPC-1)	42 µl	5 x 42 µl	-80°C
Recombinant KRAS Antibody	15 µl	44 µl	-20°C
Anti-rabbit HRP-conjugated IgG	15 µl	15 µl	-20°C
Protease Inhibitor Cocktail (PIC)	2 x 105 µl	1.2 ml	-20°C
5X Lysis Binding Buffer	4 ml	22 ml	4°C
10X Wash Buffer AM2	2 x 25 ml	2 x 125 ml	4°C
10X Antibody Binding Buffer AM2	2.5 ml	12.5 ml	4°C
Chemiluminescent Reagent	2 ml	10 ml	4°C
Reaction Buffer	4 ml	20 ml	4°C
96-well assay plate	1 ea	5 ea	4°C
Plate sealer	2 ea	10 ea	4°C

Additional materials required

- 96 well microplates
- 5 or 10 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 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

Buffer Preparation and Recommendations

Preparation of 5X Complete Lysis Binding Buffer

(For In-well Lysis in cell culture plate)

Preparation of 5X Complete Lysis Binding Buffer is required for in-well lysis without cell wash. For a 96 well culture plate, prepare a total of 3.0 ml 5X Complete Lysis Binding Buffer by mixing 2.85 ml 5X Lysis Binding Buffer with 150 µl Protease Inhibitor Cocktail. Protease inhibitors lose their activity after 24 hours once diluted, so make the 5X Complete Lysis Binding Buffer the day of use. The remaining amount should be discarded if not used in the same day.

Preparation of 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 RBD-Raf-RBD protein to the ELISA Plate. For a 96 well ELISA, prepare 5.0 ml 1X Complete Lysis Binding Buffer by mixing 50 µl Protease Inhibitor Cocktail, 1.0 ml 5X Lysis Binding Buffer, and 3.95 ml distilled water. 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 in the same day.

GST-Raf-RBD

The GST-Raf-RBD contains a RAS Binding Domain and is used to capture activated RAS on the glutathione-coated plate. 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. For a 96 well plate prepare 5.0 ml GST-Raf-RBD Solution by adding 100 µl GST-Raf-RBD to 4.9 ml 1X Complete Lysis Binding Buffer. Invert 10 times to mix. Discard any unused diluted GST-Raf-RBD.

Preparation of 1X Wash Buffer

For each 96 well ELISA prepare 300 ml of 1X Wash Buffer by mixing 30 ml of 10X Wash Buffer with 270 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 10X Wash Buffer AM2 may form clumps. If this occurs, homogenize the buffer by mixing gently.

Preparation of 1X Antibody Binding Buffer

For each 96 well ELISA prepare 13 ml 1X Antibody Binding Buffer by mixing 1.3 ml of 10X Antibody Binding Buffer with 11.7 ml distilled water. Mix gently to avoid foaming. Discard any remaining 1X Antibody Binding Buffer after use. If the BSA contained in the 10X Antibody Binding Buffer forms clumps, homogenize the buffer by warming to room temperature and inverting gently several times.

Diluted KRAS Antibody

The primary KRAS antibody recognizes human KRAS. Spin vial briefly in a microcentrifuge before opening to collect contents to the vial bottom. The supplied antibody will be diluted 1:1,000 in 1X Antibody Binding Buffer. For a 96 well plate, prepare 6.0 ml of Diluted KRAS Antibody by mixing 6.0 µl Recombinant KRAS Antibody with 6 ml 1X Antibody Binding Buffer. Invert 10 times to mix. If not using the entire vial of Recombinant KRAS antibody at one time we suggest aliquoting into smaller volumes to avoid multiple freeze and thaw cycles.

Diluted Anti-rabbit HRP-conjugated IgG

The Anti-rabbit HRP-conjugated IgG is used as the secondary antibody to detect bound primary antibody. Spin vial briefly in a microcentrifuge before opening to collect contents to the vial bottom. The supplied antibody will be diluted 1:10,000 in 1X Antibody Binding Buffer. This dilution should be made by first performing a 1:10 dilution followed by a 1:1000 dilution. For a 96 well plate, first combine 2 µl anti-rabbit HRP-conjugated IgG with 18 µl 1X Antibody Binding Buffer. Mix by pipetting up and down. Then add 6.0 µl of this mixture to 6 ml 1X Ab Binding Buffer. Invert 10 times to mix. If not using the entire vial of Anti-rabbit HRP-conjugated IgG at one time we suggest aliquoting into smaller volumes to avoid multiple freeze and thaw cycles.

Preparation of 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. These reagents are mixed in a 1:2 ratio to create the Chemiluminescent Working Solution. For a 96 well plate, mix 1.9 ml Chemiluminescent Reagent with 3.8 ml volumes of Reaction Buffer. Invert several times to mix. The Chemiluminescent Working Solution is stable for several hours. After use, discard any remaining Chemiluminescent Working Solution.

Positive Control Lysate (AsPC-1)

The Positive Control Lysate (AsPC-1) is provided as a positive control for KRAS activation. 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 10X when used at 10 µl/well. For best results we recommend thawing it no more than 15 minutes prior to use. Avoid freeze/thaw cycles of the extract and store at -80ºC.

Quick Chart for Preparing Buffers**

** **The Quick Chart includes an excess of components to perform the assay.**

Quick Chart (Per 96 well plate)

Reagents	Components	Volume
5X Complete Lysis Binding Buffer (For In-well Cell Lysis)	Protease Inhibitor Cocktail 5X Lysis Binding Buffer **Total**	150 µl 2.85 ml **3.00 ml**
1X Complete Lysis Binding Buffer (For ELISA Assay)	Protease Inhibitor Cocktail 5X Lysis Binding Buffer Distilled water **Total**	50 µl 1.0 ml 3.95 ml **5.0 ml**
GST-Raf-RBD for coating plate	GST-Raf-RBD 1X Complete Lysis Binding **Total**	100 µl 4.9 ml **5.0 ml**
1X Wash Buffer (can store for 1 week, 4C)	10X Wash Buffer Distilled water **Total**	30 ml 270 ml **300 ml**
1X Antibody Binding Buffer	10X Antibody Binding Buffer Distilled water **Total**	1.3 ml 11.7 ml **13 ml**
Diluted KRAS Antibody (1/1000 dilution)	Recombinant KRAS Antibody 1X Antibody Binding Buffer **Total**	6.0 µl 6.0 ml **6.0 ml**
Anti-rabbit HRP-conjugated IgGPre-Dilution	Anti-rabbit HRP-conjugated IgG 1X Antibody Binding Buffer **Total**	2 µl 18 µl **20 µl**
Diluted Anti-rabbit HRP-conjugated IgG	Anti-rabbit HRP- conjugated IgG Antibody Pre-Dilution 1X Ab Binding Buffer **Total**	6.0 µl 6.0 ml **6.0 ml**
Chemiluminescent Working Solution	Chemiluminescent Reagent Reaction Buffer **Total**	1.9 ml 3.8 ml **5.7 ml**

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 for 24 hours prior to use. Prepare the 5X Complete Lysis Binding Buffer, 1X Complete Lysis Binding, 1X Wash Buffer and 1X Antibody Binding Buffer as described in the section Buffer Preparation and Recommendations. A multi-channel pipette or HandyStep Repeating pipette and reagent reservoirs may be used for dispensing all reagents.

The protocol below is for the in-well lysis workflow. Alternatively, whole cell extracts can be prepared 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.

Step 1: Treatment of Cells

The number of seeded cells and incubation period should be optimized for each cell line. It is recommended that a range of 5 x 10^3 cells to 1.6 x 10^6 cells be used per well. After seeding cells, an incubation of at least 2 days is recommended to stabilize cultured cells before treatment. We suggest 3-4 replicates per experimental condition.

Treat the cells with 100 µl of media containing KRAS 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 KRAS activation/inhibition will be complete. Then proceed with **Step 3: In-well Cell Lysis**.

Step 2: Binding GST-Raf-RBD to Plate

1. Thaw the GST-Raf-RBD on ice immediately before use. For a full plate mix 100 µl GST-Raf-RBD with 4.9 ml 1X Complete Lysis Binding Buffer. Invert 10 times to mix. Dispense 50 µl GST-Raf-RBD mixture into each well.
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).
3. 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. Alternatively, a microplate washer may be used.

Step 3: In-well Lysis Cell Lysis

4. Add 25 µl 5X Complete Lysis Binding Buffer to each well of the culture plate using a multichannel pipettor. Mix by pipetting up and down 3-5 times.

   NOTE: Alternatively, culture media can be removed, and wells washed with 200 µl PBS, before adding 125 µl 1X Complete Lysis Binding Buffer to each well.

5. Firmly seal the plate. Place the sealed plate on ice for 15 minutes. For some cell lines, lysis may be improved by placing the plate on a microplate shaker at 100-120 rpm and 4°C for 15 minutes.
6. Carefully remove the plate seal. For the assay, 50 µl of lysed whole cells from each well will be transferred to the ELISA plate. Be sure the lysed cells are resuspended before the sample is transferred. Cell lysates can be stored frozen at -80 C before using in the ELISA. However, a reduction in the ELISA signal may be observed.

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 3-5 times before the sample is transferred.

Positive control wells: Thaw the Positive Control Lysate (AsPC-1) on ice for no more than 15 minutes prior to use. Mix 10 µl of extract with 40 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 only per well.

7. Cover the plate and incubate for 1 hour at room temperature with mild agitation (100 rpm on a rocking platform).
8. 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. Alternatively, a microplate washer may be used.

Step 5: Binding of primary antibody

9. Add 50 µl diluted KRAS antibody (1:1000 dilution in 1X Antibody Binding Buffer) to wells.
10. Cover the plate and incubate for 1 hour at room temperature without agitation.
11. 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. Alternatively, a microplate washer may be used.

Step 6: Binding of secondary antibody

12. Add 50 µl diluted HRP antibody (1:10,000 dilution in 1X Antibody Binding Buffer) to all wells being used.
13. Cover the plate and incubate for 1 hour at room temperature without agitation.
14. During this incubation, place Chemiluminescent Reagent and Reaction Buffer at room temperature.
15. 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. Alternatively, a microplate washer may be used.

Step 7: Chemiluminescent detection

16. Add 50 µl room-temperature Chemiluminescent Working Solution to all wells being used. Read chemiluminescence using a luminometer. Readings should be taken within 15 minutes to minimize changes in signal intensity. 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 (without in-well lysis) 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 and wash with 5 ml ice-cold PBS.
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 Bradford-based assay.
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.

Section B. 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 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	Perform antibody titration to determine optimal working concentration. Start by using 1:1,000 for primary antibody and 1:10,000 for secondary antibody. The sensitivity of the assay will decrease. Check calibration of pipettes. Background in this assay directly correlates with antibody concentration.
No signal or weak signal in sample wells	Not enough extract per well	Increase number of cells seeded in the culture plate.
	KRAS is poorly activated or inactive	Perform a time course for KRAS activation in the studied cell line.
	Extracts are not from correct species	Refer to cross-reactivity information on page 3.

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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.

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