V 1.6

RAS GEF Exchange Assay Biochem Kit

Cat. # BK101

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Manual Contents

I: Introduction

The Ras proteins are small GTPases that are important for regulating signal transduction. The three RAS isoforms N-Ras, H-RAS, and K-RAS are a group of small GTPases that serve as binary switches cycling between inactive (GDP-bound) and active (GTP-bound) states (1,2). Switch-I and switch-II form the guanosine nucleotide binding pocket and also form the binding interfaces for the regulatory proteins guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs) (1,2). These molecular switches for the RAS proteins are tightly regulated and are predominantly found in the inactive state, whereas the cancer -associated mutations trigger RAS into a constitutively active state.

The *RAS* mutations are found in approximately 19% of patients, where *KRAS* is the most oncogenic form of the *RAS* genes (3). *KRAS* can undergo alternative splicing to produce KRAS-4A and KRAS-4B, two variants that differ in the residues at the C-terminus, known as the hypervariable region. Approximately 99% of the missense mutations for the RAS isoforms are localized to the three-positions of glycine-12 (G12), glycine-13 (G13), and glutamine-61 (Q61) localized in the P-loop and switch-II regions of K-RAS4B (4) (**Figure 1**). These mutations trigger RAS into a constitutively active state by decreasing GAP-induced hydrolysis and/or altering the kinetic rates of nucleotide exchange (5).

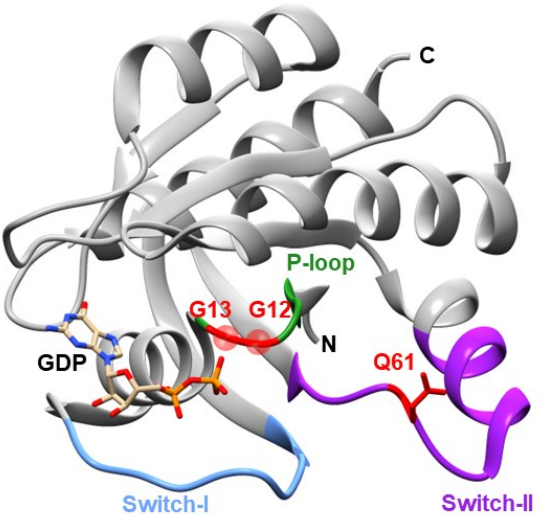

Figure 1. Crystal Structure of KRAS-4B. The crystal structure of KRAS-4B residues 1-169 (PDB ID: 4OBE). The switch region switch-I (blue residues 30-40), switch-II (magenta residues 58-72), and P-loop (green residues 10-14) are shown on the structure. Common cancerous missense mutations at amino acids G12, G13, and Q61 are highlighted in red. The structure was created with Chimera (6).

I: Introduction (Continued)

Mutant KRAS was once considered an “undruggable” target. Recently, several drugs are in clinical trials that irreversibly targets the KRAS G12C mutation in cancers (6). However, compounds that target other KRAS missense mutants or RAS isoforms remain elusive. Recently developed fluorescence analogs of guanine nucleotides allow scientists to take advantage of fluorescence-based assays to measure the spectroscopic differences between bound and unbound fluorescent analogs to guanine nucleotides and resulting in the ability to monitor nucleotide exchange of small GTPases (7, 8). Once bound to GTPases, the emission intensity of the fluorophore increases dramatically (Fig. 2). Therefore, enhancement of fluorescent intensity in the presence of small GTPases and GEFs will reflect the respective GEF activities of known or unknown proteins.

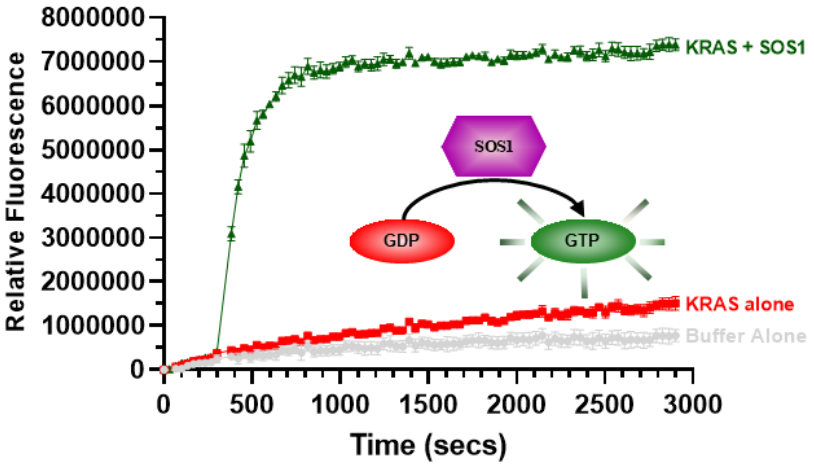

Figure 2. KRAS-4B and SOS1 exchange assay *Method: The small GTPases KRAS-4B (Cat# CS-RS03) and SOS1 (Cat# GE02) were expressed as His-tagged proteins in E. coli and purified on a nickel affinity column. The reactions were conducted in a 96-well black flat bottom half area plate (Corning Cat# 3686) format (100* µl *reaction volumes). Each reaction contains 2 µM KRAS-4B, 20 mM Tris pH 7.5, 50 mM NaCl, 10 mM* $MgCl_2$*, and 1.5 µM BODIPY FL GTP with or without 0.8 µM human SOS1(Exchange domain) protein. Reactions were measured in a SpectraMax iD5 fluorimeter (*λ*ex= 485 nm,* λ*em= 535nm). Readings were taken at 20°C every 30 seconds for a total reaction time of 30 minutes. The data shown are the averages of three experiments with error bars representing SD.*

Note: BODIPY FL GTP is a trademark of ThermoFisher Inc. MA, USA.

I: Introduction (Continued)

Cytoskeleton Inc. has developed a N-MAR-GTP fluorophore-based RAS-GEF assay suitable for both 96-well and 384-well formats (9). This assay can be utilized for multiple research purposes, such as identifying GEF or GTPase activators/inhibitors, characterizing GEFs, testing the activity of mutant GTPases, and discovering novel GEF and GTPase partners in a high-throughput screen format. (Fig. 3). This kit contains human KRAS-4B protein and the GEF domain of SOS1 as a positive control (Fig. 2). KRAS-4B cancer relevant mutant, other GTPases, or GEFs can be purchased at Cytoskeleton Inc. for analysis by monitoring N-MAR-GTP exchange.

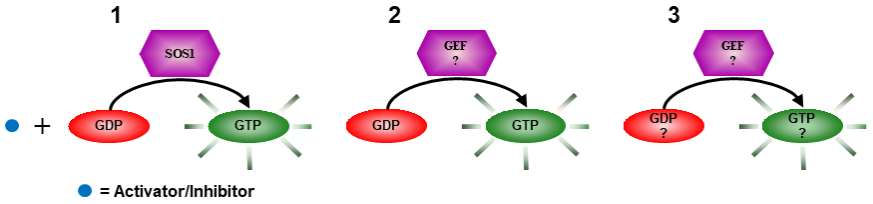

Figure 3. GTPase, GEF, and compound combinations

1) The RAS-GEF assay allows users to identify activators or inhibitors towards SOS1, KRAS-4B, GEF, or other GTPases.
2) This kit may also test mutant SOS1 GEFs or identify novel GEF proteins.
3) Analyze your choice of GEF and GTPase combinations using the exchange buffer (Cat# EB03) provided with this kit and KRAS-4B/SOS1 as a positive control.

References for Introduction

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3) Prior, I.A., et al., *Cancer Res.* **80**, 2969-2974 (2020)
4) Prior, I.A., et al., *Cancer Res.* **72(10)**, 2457-2467 (2012)
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6) Pettersen, E.F., et al., *J. Comput. Chem.*, **25**, 1605-1612 (2—4)
7) Hong, D.S., et al., *N. Engl. J. Med.*, **383**, 1207-1217 (2020)
8) Cheng, L., et al., *Mol. Cell. Biol.,* **22**, 6895-6905 (2002)
9) Rossman, K. L., et al., *EMBO J.*, **21**, 1315-1326 (2002)
10) McEwen, D.P., et al., *Anal. Biochem.* **291**, 109-117 (2001)

II: Important Technical Notes

Carefully read the following technical notes before beginning the assay

Exchange assay reagents

1) This kit contains sufficient purified GTPases and N-MAR-GTP to carry out approximately 100 reactions with 36 control reactions of 100 µl volume using a black flat-bottom 96-well half area plate (Corning plate Cat# 3686) and approximately 660 reactions with 230 control reactions per GTPase can be achieved if a 384-well black round bottom plate (Corning Cat# 4514) is used. DO NOT USE CLEAR PLATES SINCE THAT WILL GIVE SIGNIFICANT BACKGROUND NOISE.
2) The wild-type KRAS-4B protein is included in this kit. Other KRAS-4B mutants can be analyzed with this kit and purchased separately from Cytoskeleton Inc. (see **Section VII**).
3) Many of the reagents in this kit require reconstitution and division into convenient experiment-sized aliquots. It is important to carry out the aliquoting step as multiple freeze/thaw cycles of some reagents (for example, exchange buffer and purified protein) may result in the inactivation of the reagents. It is strongly recommended to store the exchange buffer (Cat# EB03) without exposing it to light for a long period of time.

Assay optimization

The exchange assay kit has been developed to characterize and examine SOS1 or other guanine nucleotide exchange factors (GEFs) with KRAS-4B. For example, using this kit as outlined in the introduction (Section I) will result in a max slope value of 5.2×10^{-3} mol N-MAR-GTP loaded per mol KRAS-4B per second, consistent with published data. **The exchange assay should be performed at 20°C. A higher temperature may cause a higher intrinsic (no GEF) rate, influencing signal-to-noise ratio significantly.** It should be noted, however, that optimization of the exchange assay may be needed for any given GEFs. Please refer to **Appendix I** for troubleshooting.

Several parameters may particularly affect GEF protein activity

1) Temperature. An exchange reaction at 20°C is required. Besides, lower or higher temperatures may cause significant changes in signal-to-noise ratio, different GEFs may require a different optimal temperature for their normal *in vitro* exchange activity.
2) Protein concentration. A titration of the GEF of interest should be performed to achieve optimal results.
3) Exchange buffer conditions. Although the condition of the reaction buffer in this kit has been optimized, it could be necessary to optimize a particular GEF assay by adjusting the salt concentrations (25-500 mM) and the pH (6.0-8.3) using appropriate buffers such as MES, PIPES, and Tris.
4) Control reactions. Including control reactions in the assay is essential, particularly if your GEF of interest is impure. Control reactions are discussed in **Section V**.

II: Important Technical Notes

Measurement type:	Character	Contents
Measurement type:	Kinetic	60 cycles of 1 reading per 30 seconds
Fluorescence wavelengths: *Note: Wavelengths are provided for assays run on monochromatic fluorimeters.*	Filter-based Ex. Em. Monochromatic Ex. Em.	 485 nm ± 20 nm 535 nm ± 20 nm 485 nm 515 nm
Gain:		80 on a scale of 0-120, where 120 is the highest
Reads per well:	Flashes	5
Fluorescence reading from:	Top	
Integration:		0-40 µs
Shaking:	Med, orbital	5 seconds (first read only)
Plate-type:	Greiner	GRE384fb or GRE96fb (flat, black). Standard template on TECAN.
Max slope:	AFU/ second	12 readings

Instrumentation

The fluorescence reaction is based upon an excitation at 485 nm and an emission at 515 nm on a monochromatic fluorometer. Therefore, the filter-based fluorimeter should be set at an excitation filter wavelength of 485 +/- 20 nm and an emission filter wavelength at 535 +/- 20 nm. The bandwidth of the filter should be no more than 25 nm, or you may experience significant background noise and reduced sensitivity of the assay. Non-filter based monochromatic fluorometers usually have +/- 2 nm bandwidth, so there is no problem with bandwidth when using these machines. The fluorimeter should be at 20°C and set on kinetic mode; it is recommended to take a reading once every 30 seconds for at least 60 cycles (30 minutes total). There is no need to elect a blank well, as the reaction without GEFs will serve as a background control.

Most of the work in the design of this assay was developed on the Tecan GmbH machine called SpectroFluor Plus and SpectraMax iD5. The SpectroFluor Plus instrument is a filter-based fluorimeter that can detect pmoles of fluorescein. A similar fluorimeter or a SpectraMax ID5 spectrometer is recommended to use for this assay.

Uses and applications for this kit:

1. Determination of the activity of unknown GEFs.
2. Biochemical characterization of small GTPases and their associated GEFs.
3. Examination of the regulation of GEF activity by different cofactors or protein domains.

4. Screening mutant proteins of either GEFs or GTPases for activity and substrate specificity.
5. Identify of GEF inhibitors in an HTS (high throughput screen) format. Please inquire about significant discounts on large quantities of reagents in this kit.

III: Kit Contents

KIT COMPONENT	DESCRIPTION
Exchange Buffer (2x) (Part# EB03)	One bottle, lyophilized. When reconstituted: 40 mM Tris pH 7.5, 100 mM NaCl, 20 mM $MgCl_2$, 3.0 µM N-MAR-GTP.
His-KRas4B protein (Cat# CS-RS03)	Three tubes, lyophilized. Contains 100 µg of purified N-terminal His-tagged KRas-4B protein.
His-hSOS1-His protein (Cat# GE02)	Two tubes, lyophilized. Contains 100 µg of purified N-terminal His-tagged human SOS1 protein (Exchange domain aa 564-1049).
384-well plate	One 384-well black half-area round bottom plate (Corning Cat# 4514)
96-well plate	One 96-well black half-area flat bottom plate (Corning Cat# 3686)

IV: Things to do Prior to Beginning the Assay

Before beginning the assay, you will need to reconstitute several components as follows:

Kit Component	Reconstitution	Storage Conditions
Exchange Buffer (2x)	1) Dissolve the powder in each bottle with 5 ml of nanopure water. 2) Aliquot into 10 x 0.5 ml sizes (per bottle).	Store at -70°C. Stable for six months under these conditions.
His-KRas-4B protein	1) Briefly centrifuge the tube to make sure all the white protein powder is at the bottom of the tube. 2) Check that you can see the white powder pellet. 3) Reconstitute in 20 µl of nano-pure water to give a 200 µM (5 mg/ml) solution. 4) Aliquot into 4 x 5 µl sizes. 5) Snap freeze in liquid nitrogen. **Note:** Follow the same reconstitu-tion instructions for KRAS4B mu-tants.	Same as above.
His-SOS1 protein	1) Briefly centrifuge both tubes to make sure all the white protein powder is at the bottom of the tube. 2) Check that you can see the white powder pellet. 3) Reconstitute each tube in 33 µl of nanopure water and combine to give 66 µl of a 50 µM (3 mg/ml) solution 4) Aliquot into 8 x 8 µl sizes. Snap freeze in liquid nitrogen. **Note:** The SOS2 exchange domain (Cat# CS-GE08) can also be pur-chased through Cytoskeleton.	Same as above.

V: Protocol

The following protocols are for either a 96-well or 384-well plate format (Corning Cat# 3686 and 4514, respectively). The volume can be adjusted by ratio using different volume plates. The exchange reaction starts with all of the reaction components minus SOS1 or GEF of interest. SOS1 or GEF of interest is added after 5-10 readings to examine its exchange activity.

Reaction protocol for 96-well plate

1) Set up and test the plate reader and kinetic parameters, and prepare protein or other samples before starting.
2) Thaw an aliquot of 2x Exchange Buffer in a room-temperature water bath for 1 minute. Keep at ROOM TEMPERATURE and protect from light (foil wrap works well for this).
3) Thaw one 5 µl aliquot of wild-type KRAS-4B (Cat# CS-RS03) or KRAS-4B mutant (200 µM) depending on which GTPase is being tested (Please refer to **Section VII** for more GTPase choices), by placing in a room temperature water bath for 1 minute. Dilute to 50 µM by addition of 15 µl of nanopure water and place on ice.
4) Thaw one 8 µl aliquot of positive control protein human SOS1 (50 µM) or your own GEF by placing them in a room temperature water bath for 1 minute. Dilute to 8 µM by addition of 42 µl Nanopure water. Place on ice.

ADJUST THE ALIQUOTED VOLUME BASED ON THE NUMBER OF REACTIONS AND THE KIND OF PLATE TO BE USED. The recommended working concentration of GEFs is 0.2 – 2 µM (consider the percent purity of your GEF protein in this calculation). NOTE: we use 0.8 µM GEF in the standard hSOS1 assay (see Figs. 1 – 3); you may want to titrate the concentration of GEF in your particular experimental setup.

5) Add the following components together at ROOM TEMPERATURE and mix well by pipetting or gentle vortex:

Exchange reaction mix:	**One 96-well reaction:**
2x Exchange Reaction Buffer (Part# EB03)	50 µl
KRas-4B (50 µM) (Cat# CS-RS03) or mutant (**See Section VII)**	4 µl
Nanopure water	36 µl

6) Adjust the volume based on the plate being used. The recommended volumes here are based upon a total volume of 100 µl (96-Well half area plate, Corning Cat# 3686)
7) The reaction mix is now ready for the fluorescence reading (λ_{Ex} = 485 nm, λ_{Em} = 535 nm). Aliquot to the assigned well and place the plate in the fluorimeter.

WE STRONGLY RECOMMEND THE OPERATION TEMPERATURE FOR THE FLUORIMETER SHOULD BE 18-22°C. A HIGHER TEMPERATURE MAY CAUSE A HIGHER INTRINSIC RATE AND THUS INFLUENCE THE SIGNAL/NOISE RATIO SIGNIFICANTLY.

8) After 5 readings (150 seconds), pipette 10 µl of either your GEF, SOS1 protein (8 µM) or nanopure water (intrinsic control) in respective wells and immediately pipette up and down twice and start reading.

IT IS RECOMMENDED TO ADD GEF PROTEINS (OR WATER) USING A MULTICHANNEL PIPET TO ENSURE ALL REACTIONS BEGIN SIMULTANEOUSLY. IT IS IMPORTANT TO KEEP THIS PROCESS AS SHORT AS YOU CAN TO GET A SMOOTH CURVE. KEEP READING UNDER THE SAME CONDITIONS.

9) Save the readings after the kinetic protocols are finished. The exchange rate can be calculated by reducing the data to max slope with the software accompanying the plate reader. The exchange curve can be achieved by exporting raw data to Microsoft Excel. Examples using SOS1 are shown in **Figure 2**. To calculate the specific activity, please read **Appendix II.**

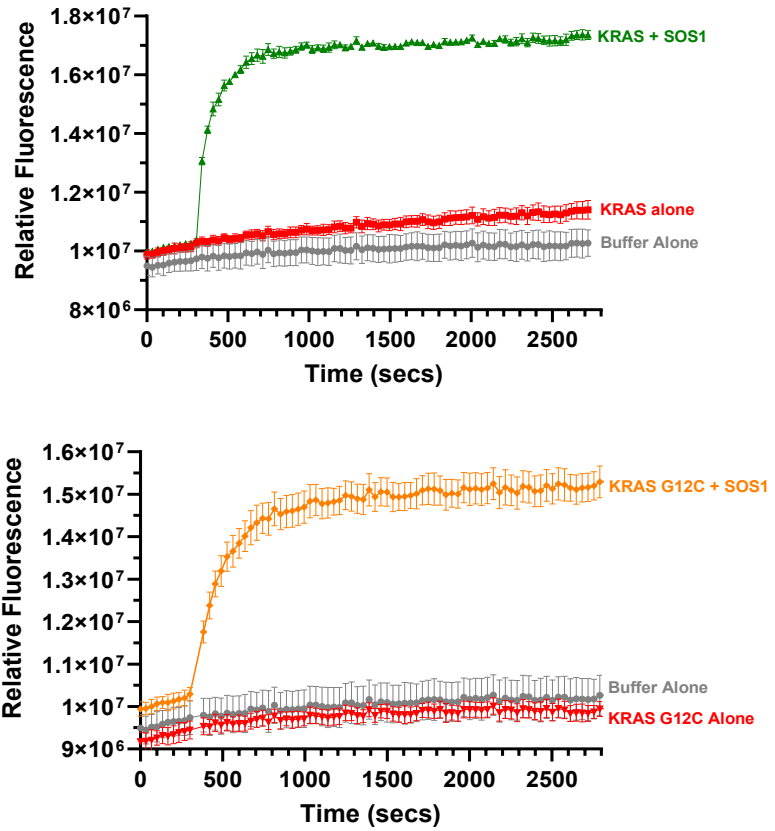

Figure 2. SOS1 exchange activity for KRAS-4B and KRAS-4B G12C (Cat# CS-RS14) in 96-well half-area plate format. The raw data shown are the average points (n=3) with error bars performed on a SpectraMax iD5.

Reaction protocol for 384-well plates (Corning Cat# 4514) - Highly recommended for high-throughput screening

1) Set up and test the plate reader and kinetic parameters, and prepare protein samples or compounds before starting.
2) Thaw the relative amount of respective GTPase, GEF, and drug solution (for high throughput screen of GEF inhibitors only) by placing it in a room temperature water bath for 1 minute. Place on ice.
3) Thaw a 2x Exchange Buffer aliquot by placing it in a room temperature water bath and leaving it at room temperature, protected from light.
4) Thaw one 5 µl aliquot of wild-type KRAS-4B (Cat# CS-RS03) or KRAS-4B mutant (200 µM) depending on which GTPase you want to test (Refer to **Section VII** for more GTPase choices). Dilute to 15 µM by addition of 60 µl of Nanopure water and place on ice.
5) Thaw one 8 µl aliquot of positive control protein human SOS1 or your own GEF by placing them in a room temperature water bath for 1 minute. Dilute to 4 µM by addition of 92 µl Nanopure water. Place on ice.
6) Add the following components together at ROOM TEMPERATURE and mix well by pipetting or gentle vortex:

Exchange reaction mix:	**One 384-well reaction (µl):**	**A full 384-well plate of reactions + 15% pipetting losses (µl):**
SOS1 (4 µM)	3	1350
KRas-4B (15 µM) (Cat# CS-RS03) or mutant (**See Section VII)**	2	900
Nanopure water	2.5	1150

ADJUST THE VOLUME BASED ON THE PLATE YOU ARE USING. The recommended volume here is based upon a total volume of 15 µl (384-well black round bottom half area plate, corning Cat# 4514). The recommended working concentration of GEFs is 0.2 – 1 µM (Consider the percent purity of your GEF protein in this calculation). NOTE: it is recommended to use 0.8 µM GEF in the standard SOS1 assay (see Figs. 1 – 3); you may want to titrate the concentration of GEF in your experimental setup.

For HTS only: THE RECOMMENDED CONCENTRATION OF DRUG SOLUTION IS 10 µM – 100 µM . We recommend you titrate your drug solution for optimal results. Generally, a 30 µM – 50 µM concentration is good for initial screening.

7) The reaction mix is now ready for the fluorescence reading (λ_{Ex} = 485 nm, λ_{Em} = 535 nm). Pipette 7.5 µl exchange reaction mix to the assigned wells and place the 384-well round bottom black plate in the fluorimeter.

WE STRONGLY RECOMMEND THE OPERATION TEMPERATURE FOR THE FLUORIMETER SHOULD BE AROUND 20°C. A HIGHER TEMPERATURE MAY CAUSE HIGHER INTRINSIC RATE AND THUS INFLUENCE THE SIGNAL/NOISE RATIO SIGNIFICANTLY.

8) After 5 readings (150 seconds), add 7.5 µl of the 2x Exchange Reaction Buffer (Part# EB03) to respective wells and immediately resume reading.

IT IS RECOMMENDED TO ADD GEF PROTEINS (OR WATER) USING A MULTICHANNEL PIPET TO ENSURE ALL REACTIONS BEGIN SIMULTANEOUSLY. IT IS IMPORTANT TO KEEP THIS PROCESS AS SHORT AS YOU CAN TO GET A SMOOTH CURVE. KEEP READING UNDER THE SAME CONDITIONS.

9) Save the readings after the kinetic protocols are finished. The exchange rate can be calculated by reducing the data to max slope with the software that accompanying the plate reader. The exchange curve can be achieved by exporting raw data to Microsoft Excel. Examples using SOS1 are shown in **Figure 3.** To calculate the specific activity, please read **Appendix II**.

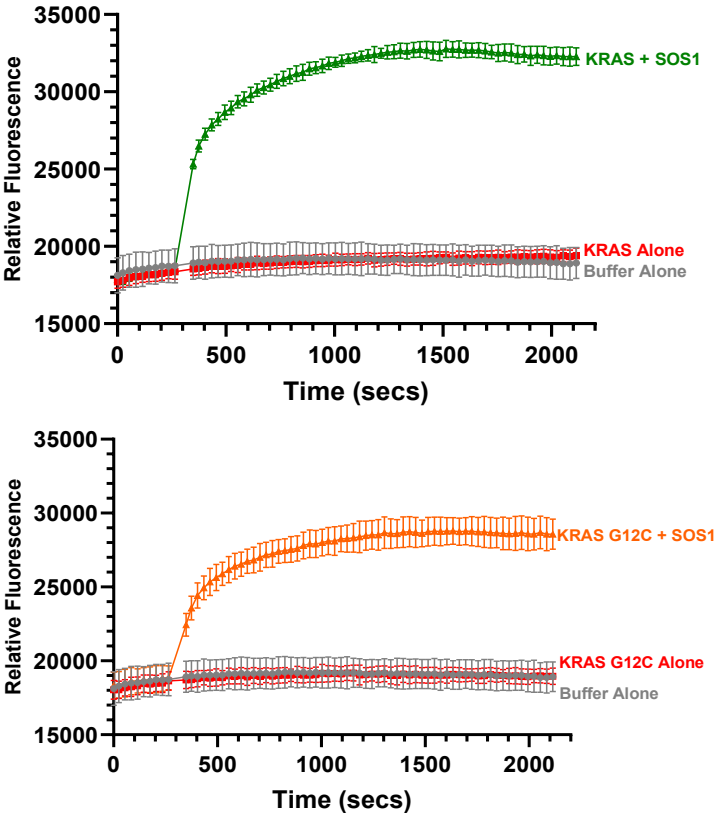

Figure 3. SOS1 exchange activity for KRAS-4B and KRAS-4B G12C (Cat# CS-RS14) in 384-well half-area plate format. The raw data shown are the average points (n=3) with error bars performed on a Tecan Spectrofluor plus

VI: References

Citations for BK101

Check the website for updates to product citations.

www.cytoskeleton.com/BK101

VII: Related Products

Catalog Number	Item	Ras Subfamily	Quantity
CS-RS01	H-Ras protein (His-tagged)	Ras	1 x 100 µg
CS-RS02	N-Ras protein (His-tagged)	Ras	1 x 100 µg
CS-RS03	K-Ras4B protein (His-tagged)	Ras	1 x 100 µg
CS-RS04	K-Ras4B G12V protein (His-tagged)	Ras	1 x 100 µg
CS-RS06	K-Ras4B G13D protein (His-tagged)	Ras	1 x 100 µg
CS-RS07	K-Ras4B G13S protein (His-tagged)	Ras	1 x 100 µg
CS-RS08	K-Ras4B K128A protein (His-tagged)	Ras	1 x 100 µg
CS-RS09	K-Ras4B Q61P protein (His-tagged)	Ras	1 x 100 µg
CS-RS10	K-Ras4B R135A protein (His-tagged)	Ras	1 x 100 µg
CS-RS11	K-Ras4B G12S + I36N protein (His-tagged)	Ras	1 x 100 µg
CS-RS12	K-Ras4B G12D + D38A protein (His -tagged)	Ras	1 x 100 µg
CS-RS13	K-Ras4B G12D protein (His-tagged)	Ras	1 x 100 µg
CS-RS14	K-Ras4B G12C protein (His-tagged)	Ras	1 x 100 µg
RC03	RhoC protein (His-tagged)	Rho	1 x 100 µg
CS-GE01-A CS-GE01-C	Human Dbs protein (His-tagged)	Rho	1 x 50 µg 3 x 50 µg
CS-GE02 CS-GE02-XL	SOS1 GEF protein (Exd exchange domain, aa564-1049, His-tagged)	Ras	1 x 100 µg 1x 1 mg
CS-GE03	RasGRF1 GEF protein (Cdc25 exchange domain, aa 1038-1270, MBP-tagged)	Ras	1 x 100 µg
CS-GE04	Tiam1 GEF protein (DHPH exchange domain, aa 1040-1406 MBP -tagged)	Rho	1 x 100 µg
CS-GE05	VAV1 GEF protein (DHPHC1 exchange domain, Y174D mutant, aa 168-522, His-tagged)	Rho	1 x 100 µg
CS-GE06	VAV2 GEF protein (DH exchange domain, aa 189-374, His-tagged)	Rho	1 x 100 µg
CS-GE07	ARNO GEF protein (Sec7 exchange domain, aa 31-267, His-tagged)	Arf	1 x 100 µg
CS-GE08	SOS2 GEF protein (Exd exchange domain, aa563-1051, His-tagged)	Ras	1 x 100 µg
BK008	Ras activation assay biochem kit	Ras	25 assays
BK034	Cdc42 activation assay biochem kit	Rho	25 assays
BK035	Rac1 activation assay biochem kit	Rho	25 assays
BK036	Rho activation assay biochem kit	Rho	25 assays
BK100	Rho GEF exchange assay	Rho	25 assays

Note: More GTPases are available online at https://www.cytoskeleton.com/small-g-proteins/proteins.

Cytoskeleton, Inc. has extensive experience in providing protein purification services for recombinant and native source proteins. Please visit https://www.cytoskeleton.com/custom-services to learn about these services for producing proteins that are currently not in our catalog.

Appendix I: Troubleshooting

Observation	Possible cause	Remedy
1. No increase in fluorescence after adding SOS1.	1. The sensitivity of fluorescence spectrophotometer set too low . 2. Incorrect labeling of tubes. 3. Inactive proteins.	1) Increase sensitivity by increasing emission gain or increasing intensity of excitation. 2) Repeat the experiment. 3) Follow the correct storage procedure.
2a. During the assay, the increase in fluorescence is too slow for the tested GEF protein.	1. GEF concentration is too low or GEF is a weak exchanger for this GTPase. 2. The excitation light is too intense. 3. The purity of GEF protein is too low. 4. GTPase concentration is too low.	1) Increase GEF concentration or titrate the GEF concentration. 2) Reduce excitation light intensity. 3) Increase GEF purity. 4) Increase GTPase concentration.
2b. During the assay, the increase in fluorescence is too slow for positive control Dbs.	1. The excitation light is too intense. 2. GTPase concentration is too low.	1) Reduce light intensity. 2) Increase GTPase concentration.
3. During the assay, increase in fluorescence is too quick.	1. GEF concentration is too high. 2. GTPase concentration is too high. 3. GEF is a strong exchanger for specific GTPase.	1) Decrease GEF concentration. 2) Decrease GTPase concentration.
4. During the assay, an increase in fluorescence is not reproducible.	1. Inconsistent preparation of reaction mixture. 2. GEF protein is not stable due to poor purity.	1) More consistent technique, increase pipetting accuracy, or test machine for signal stability using buffer alone. 2) Increase the purity of the protein by optimizing the purifying process, e.g., reducing the proteases.

Appendix I: Troubleshooting

Observation	Possible cause	Remedy
5. During GEF assay increase in fluorescence is low.	1. Concentration of GEF too low 2. The GEF is a weak exchanger for a specific GTPase.	1) Increase GEF concentration. 2) Perform the experiment together with positive control GEF.
6. Buffer components of the reaction interfere with the activity of the test protein or compound.	1. Tris-HCl 2. pH 3. $MgCl_2$ 4. NaCl	1) Tris-HCl: Make new GEF Buffer with a different "GOOD" Buffer. 2) pH: Generally GEF can function at pH6.0-8.5. 3) $MgCl_2$: Lower the concentration to 5 mM. 4) NaCl: Adjust the concentration to 20 mM.
7. The intrinsic exchange rate is too high.	1. The reaction temperature is too high.	1) Cool down the instrument to 20°C.
8. Test GEF protein has no exchange activity.	1. Test GEF does not exchange the specific GTPase. 2. The optimized condition is not good for testing this GEF protein.	1) Try different GTPases. 2) Titrate pH etc. for optimal results.

Appendix II: Specific Activity

Two steps to calculate specific exchange rate:

1. [Max Slope (AFU/sec)] / [Basal N-MAR-GTP AFU* x N**] = A (µM/sec)
2. Exchange rate = A / 1.5*** (µmol N-MAR-GTP/µmol GTPase/sec)

* Basal N-MAR-GTP level is the start point level of fluorescence units of 1.5 µM N-MAR-GTP without GEF under our condition.

** N = quantum yield (our unpublished observations):

For KRas N = 4.5

*** This number is based upon fully N-MAR-GTP loaded GTPases.

Example:

This example calculation uses data derived from Fig 1 in this manual (Page 3):

1. [Max Slope (AFU/sec)] / [Basal N-MAR-GTP AFU* x N**] = 570 (AFU/sec) / 330000 AFU x 4.5

$= 7.7 \times 10^{-3}$ (µM/sec)

2. Therefore, Exchange rate $= 7.7 \times 10^{-3}$ (µM/sec) / 1.5 µM

Volumes cancel out,

$= 5.2 \times 10^{-3}$ (µmol N-MAR-GTP/µmol KRAS/sec)

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