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SZABO-SCANDIC HandelsgmbH

Quellenstraße 110, A-1100 Wien

T. +43(0)1 489 3961-0

F. +43(0)1 489 3961-7

mail@szabo-scandic.com

www.szabo-scandic.com

[linkedin.com/company/szaboscandic](https://www.linkedin.com/company/szaboscandic) 

Description

The KRAS Isoform B Coupled Nucleotide Exchange Assay Kit is designed for screening and profiling of KRAS Isoform B antagonists/inhibitors by monitoring the binding of an effector protein such as the Ras binding domain of Raf1, (RBD-cRaf) to KRAS. The KRAS Isoform B Coupled Nucleotide Exchange Assay Kit comes in a convenient 384-well format, with enough purified recombinant **GDP-loaded KRAS Isoform B**, GTP, exchange factor SOS1, an effector protein RBD-cRAF, assay buffer and additives for 400 reactions. With this kit, a few simple steps on a microtiter plate are required for nucleotide exchange detection. First, a sample containing GDP-loaded KRAS Isoform B is incubated with SOS1 and GTP for the nucleotide exchange. Next, RBD-cRAF is added and incubated for the effector-RAS binding. Then, acceptor and donor beads are added and incubated for detection followed by reading the Alpha-counts.

SOS1 (son of sevenless) is a guanine nucleotide exchange factor that facilitates the exchange of GDP for GTP. GDP-loaded KRAS Isoform B is in an inactive state and does not interact with the Ras-binding domain (RBD) of cRAF. SOS1 assists in the release of GDP from KRAS Isoform B so that GTP can occupy the nucleotide binding pocket. This results in a conformational change in KRAS Isoform B that permits its binding to RBD-cRAF. The KRAS Isoform B Coupled Nucleotide Exchange Assay Kit utilizes GST-tagged RBD-cRAF and His-tagged KRAS Isoform B to assay binding of KRAS to RBD-cRAF in the Alpha assay. Glutathione acceptor and Ni chelate donor beads are brought into proximal range by the binding of KRAS and RBD-cRAF, enabling the energy transfer from the donor to acceptor beads after laser excitation.

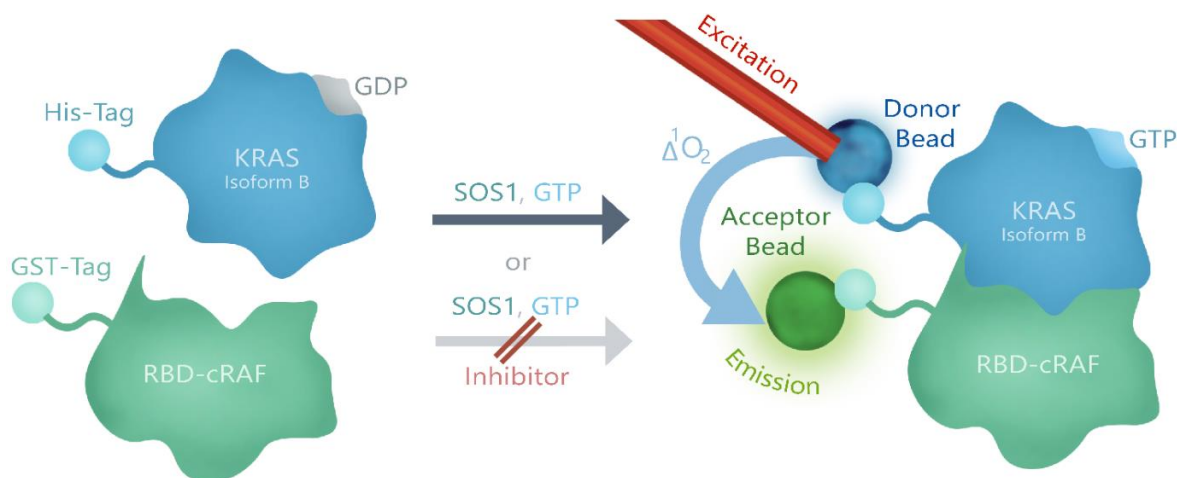


Figure 1: Illustration of the assay principle.

Background

High levels of wild-type KRAS cause a slowing of cell replication and growth, and increased apoptosis. This can be induced by cellular stress, certain types of radiation, chemical signals, and other prompts. Wild-type KRAS may also protect against mutant KRAS over-activation by dimerizing with mutant KRAS protein.

The study of differences in the behavior of the wild-type and mutated forms of KRAS is especially important since KRAS mutations are responsible for more than 30% of human cancers. Compounds that affect the nucleotide exchange (GDP to GTP) reaction provide a novel approach to the inhibition of tumor cell growth in KRAS-driven tumors. In 2021, the Food and Drug Administration granted approval for use of AMG510 (Sotorasib), a potent KRAS G12C inhibitor for non-squamous non-small cell lung cancer.

Application(s)

- Screen small molecule inhibitors or antagonists that affect KRAS Isoform B nucleotide-binding status in high throughput (HTS) applications.
- Counter-screen for the compounds that affect mutated forms of KRAS.

Supplied Materials

Catalog #	Name	Amount	Storage
101522	GDP-loaded KRAS Isoform B, His-Tag*	5 µg	-80°C
101573	SOS1, FLAG-Tag*	50 µg	-80°C
100519	RBD-cRAF, GST-Tag*	5 µg	-80°C
79861-2	GTP (10 mM)	0.5 ml	-20°C
	RBD-RAS Binding Buffer (Incomplete)	2 x 3 ml	4°C
	DTT (0.5 M)	2 x 200 µl	-20°C
79311	3x Immuno Buffer 1	4 ml	-20°C

*The concentration of the protein is lot-specific and will be indicated on the tube.

Materials Required but Not Supplied

Name	Ordering Information
AlphaLISA® Glutathione acceptor beads, 5 mg/ml	PerkinElmer #AL109C
AlphaScreen® Nickel Chelate donor beads, 5 mg/ml	PerkinElmer #AS101D
Optiplate - 384	PerkinElmer #6007290
AlphaScreen® microplate reader	
Adjustable micropipettor and sterile tips	

Storage Conditions

This assay kit will perform optimally for up to 6 months from date of receipt when the materials are stored as directed. Avoid multiple freeze/thaw cycles!

Safety

This product is for research purposes only and not for human or therapeutic use. This product should be considered hazardous and is harmful by inhalation, in contact with skin, eyes, clothing, and if swallowed. If contact occurs, wash thoroughly.

Assay Principle

AlphaLISA® immunoassays are no-wash alternatives to ELISA immunoassays using a proprietary system developed by PerkinElmer. These homogeneous assays are robust, and they are ideal for a minimal hands-on approach. The Nickel-coated Alpha donor bead binds to the His-tagged KRAS Isoform B protein, while the glutathione-coated AlphaLISA® acceptor bead binds to the GST-tag on RBD-cRAF. Glutathione acceptor and Ni chelate donor beads are brought into proximal range by the binding of KRAS Isoform B and RBD-cRAF, enabling the energy transfer from the donor to acceptor beads after laser excitation.

Contraindications

Green and blue dyes, such as Trypan Blue, absorb light in the AlphaScreen® signal emission range (520-620 nm). Avoid the use of the potent singlet oxygen quenchers such as sodium azide (NaN_3) or metal ions (Fe^{2+} , Fe^{3+} , Cu^{2+} , Zn^{2+} and Ni^{2+}). The presence of >1% RPMI 1640 culture medium leads to a signal reduction due to the presence of excess biotin and iron in this medium. MEM, which lacks these components, does not affect AlphaScreen® assays.

Assay Protocol

- All samples and controls should be tested in duplicate.
 - We recommend preincubating the GDP-loaded KRAS Isoform B with inhibitors if the inhibition mechanism is similar to AMG510; however, it is acceptable to add the GTP and SOS1 without the preincubation step.
 - The assay requires a Positive Control and a Negative Control in addition to the Test Inhibitor.
1. Prepare **Complete RBD-RAS Binding Buffer**: Add 6 μl of 0.5 M DTT to 3 ml of RBD-RAS Binding Buffer (Incomplete). Mix well.
 2. Thaw **GDP-loaded KRAS Isoform B** on ice. Briefly spin the tube containing the protein to recover the full content of the tube.
 3. Dilute **GDP-loaded KRAS Isoform B** to 3 ng/ μl in **Complete RBD-RAS binding buffer**.

*Note: The concentration of **GDP-loaded KRAS Isoform B** provided may vary. Verify the concentration of the **GDP-loaded KRAS Isoform B** written on the tube and dilute accordingly. Prepare only the amount required for the assay. Discard any unused diluted KRAS Isoform B.*

4. Aliquot any unused (undiluted) **GDP-loaded KRAS Isoform B** into single use aliquots. Store the remaining undiluted protein in aliquots at -80°C immediately.

*Note: **GDP loaded KRAS Isoform B** is very sensitive to freeze/thaw cycles. Do not re-use thawed aliquots and do not re-use the diluted protein.*

5. Add 4 μl /well of diluted **GDP-loaded KRAS Isoform B** (12 ng/well).
6. Prepare the Test Inhibitor (2 μl per well): For a titration, prepare serial dilutions at concentrations 5-fold higher than the desired final concentrations. The final volume of the reaction is 10 μl .

Without DMSO

- a. If the Test Inhibitor is water-soluble, prepare serial dilutions of the compound in **Complete RBD-RAS Binding Buffer**, 5-fold more concentrated than the desired final concentrations. For the positive and negative controls, use Complete RBD-RAS binding buffer (Diluent Solution).

Or**With DMSO**

- a. If the Test inhibitor is soluble in DMSO, prepare the test inhibitor at 100-fold the highest desired concentration in DMSO (*i.e.*, if the highest testing concentration is 50 μM , prepare a 5 mM solution in 100% DMSO). Then dilute the inhibitor 20-fold in **Complete RBD-RAS binding buffer** to prepare the highest concentration of the 5-fold intermediate solution (*i.e.*, to test at 50 μM , prepare a 250 μM intermediate solution by adding 5 μl of 5 mM inhibitor solution to 95 μl of **Complete RBD-RAS binding buffer**). The concentration of DMSO is now 5%.

- b. Prepare serial dilutions of the Test Inhibitor at 5-fold the desired final concentrations using 5% DMSO in **Complete RBD-RAS binding buffer** to keep the concentration of DMSO constant.
- c. For positive and negative controls, prepare 5% DMSO in **Complete RBD-RAS binding buffer** (vol/vol) so that all wells contain the same amount of DMSO (Diluent Solution).
Caution: Do not exceed 5% DMSO in the 5-fold intermediate solution.



The final concentration of DMSO in the reaction should not exceed 1%.

7. Add 2 µl of 5-fold intermediate serial dilutions of the Test Inhibitor to the testing wells.
8. Add 2 µl of 5% DMSO in Complete RBD-RAS binding buffer to the positive and negative control wells or add 2 µl of Complete RBD-RAS binding buffer if the Test Inhibitor was dissolved in buffer.
9. Briefly centrifuge the plate and incubate for 30 minutes at room temperature.
10. Thaw **GTP (10 mM)** and keep it on ice.
11. Thaw **SOS1** on ice. Briefly spin the tube containing the protein to recover the full content of the tube.
12. Dilute **SOS1** in Complete **RBD-RAS binding buffer** to a concentration of 120 ng/µl. *Note: The concentration of SOS1 provided may vary. Verify the concentration of the protein written on the tube and dilute accordingly.*

Caution: Prepare only the amount required for the assay. Discard any unused diluted SOS1.

13. Aliquot unused (undiluted) SOS1 into single use aliquots. Store the aliquots at -80°C immediately.

Note: SOS1 is very sensitive to freeze/thaw cycles. Do not re-use thawed aliquots and do not re-use the diluted protein.

14. Combine **GTP (10 mM)** and diluted **SOS1 (120 ng/µl)** at a 1:1 ratio (2 µl/well): N wells × (1 µl of diluted SOS1 (120 ng/µl) + 1 µl of GTP (10 mM)).
15. Initiate the exchange reaction by adding 2 µl of GTP/SOS1 mix prepared as described above to the “Test Inhibitor” and the “Positive control” wells. For the “Negative control”, add 2 µl of Complete **RBD-RAS binding buffer** only.
16. Briefly centrifuge the plate and incubate at room temperature for 30 minutes.
17. Thaw **RBD-cRAF** on ice. Briefly spin the tube containing the protein to recover the full content of the tube.
18. Dilute **RBD-cRAF** in Complete **RBD-RAS binding buffer** to a concentration of 3.6 ng/µl. *Note: The concentration of RBD-cRAF provided may vary. Verify the concentration of RBD-cRAF written on the tube and dilute it accordingly. Due to the relatively high concentration of the RBD-cRAF, an excess amount of the protein is supplied for your convenience).*

Caution: Prepare only the amount required for the assay. Discard unused diluted RBD-cRAF.

19. Aliquot the remaining unused, undiluted **RBD-cRAF** into single use aliquots. Store aliquots at -80°C immediately. *Note: **RBD-cRAF** is very sensitive to freeze/thaw cycles. Do not re-use thawed aliquots and do not re-use the diluted protein.*
20. At the end of the 30-minute incubation with SOS1/GTP, initiate the reaction by adding 2 μl of the diluted **RBD-cRAF** (3.6 ng/ μl , 7.2 ng/well) to all wells. The final reaction volume is 10 μl .
21. Briefly centrifuge the plate and incubate at room temperature for 30 minutes.

Component	Negative Control	Positive Control	Test Inhibitor
GDP-loaded KRAS Isoform B (3 ng/ μl)	4 μl	4 μl	4 μl
Test Inhibitor	-	-	2 μl
Diluent Solution	2 μl	2 μl	-
Centrifuge and incubate	30 minutes at room temperature		
GTP (10 mM)/SOS1 (120 ng/ μl) mixture	-	2 μl	2 μl
Complete RBD-RAS Binding Buffer	2 μl	-	-
Centrifuge and incubate	30 minutes at room temperature		
RBD-cRAF (3.6 ng/ μl)	2 μl	2 μl	2 μl
Centrifuge and incubate	30 minutes at room temperature		
Total	10 μl	10 μl	10 μl

22. Dilute **3X Immuno Buffer** in deionized water to prepare 1X Immuno Buffer by adding one volume of 3X Immuno Buffer to two volumes of deionized water.
23. Dilute the Glutathione Acceptor beads (PerkinElmer #AL109C) and the Nickel chelate Donor beads (PerkinElmer #AS101D) at 1:500 and 1:250 respectively in 1x Immuno Buffer (i.e., for 400 reactions, ~8 ml of the detection reagent is needed. Therefore add 16 μl of Glutathione Acceptor beads and 32 μl of Nickel Donor beads to 8 ml of 1X Immuno Buffer).



Protect your samples from direct exposure to light. Photobleaching will occur.

24. Add 20 μl of acceptor/donor beads mixture to all the wells.
25. Incubate 30 minutes at room temperature.
26. Read Alpha-counts using a compatible plate reader (PerkinElmer).

Example Results

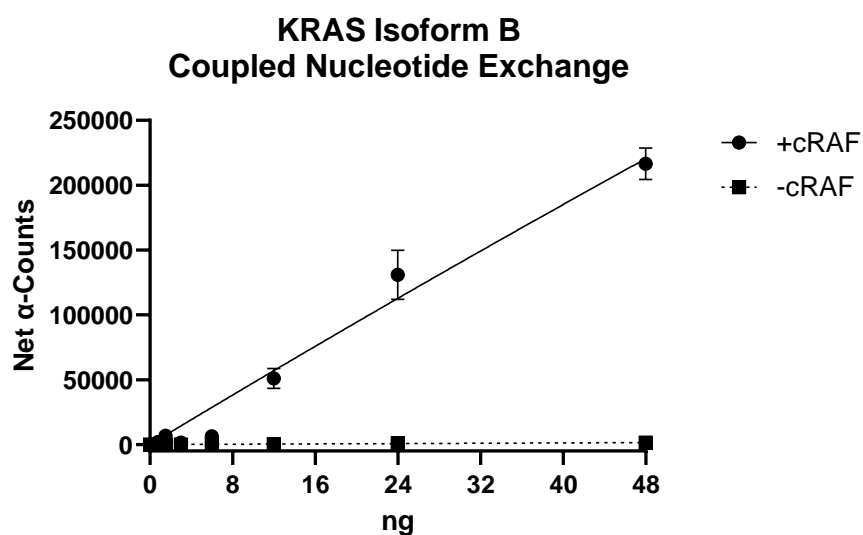


Figure 1: Nucleotide exchange of KRAS Isoform B.

The nucleotide exchange of KRAS Isoform B was evaluated in the presence or absence of cRAF.

KRAS, Isoform B, Coupled Nucleotide Exchange

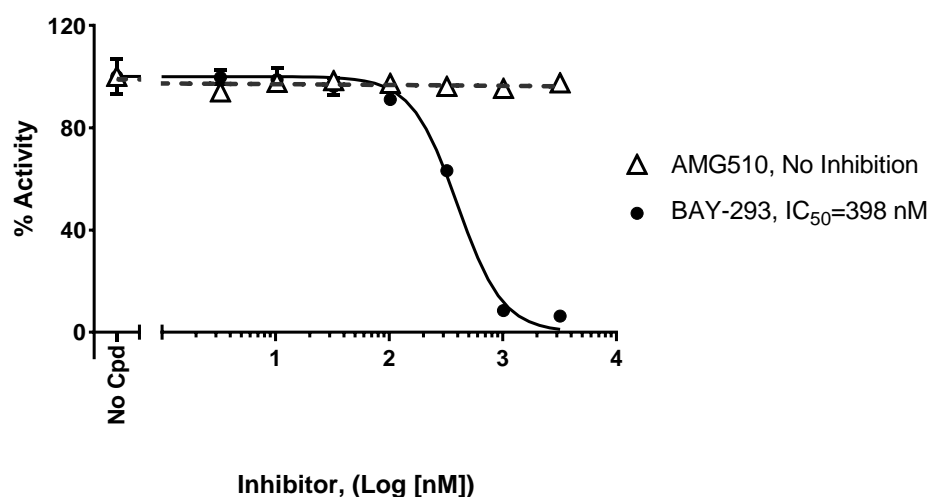


Figure 2: Effect of KRAS inhibitors on the nucleotide exchange of KRAS Isoform B.

Inhibition of the nucleotide exchange of KRAS Isoform B was evaluated in the presence of increasing concentrations of BAY-293 and AMG510 using KRAS Isoform B Coupled Nucleotide Exchange Assay Kit (BPS Bioscience #...). BAY-293 selectively inhibits the KRAS-SOS1 interaction. AMG510 is a covalent inhibitor of KRAS G12C that does not affect wild-type KRAS.

Data shown is representative. For lot-specific information, please contact BPS Bioscience, Inc. at support@bpsbioscience.com

General Considerations

Plates and Instruments: A plate reader capable of Alpha technology detection is required. We recommend using PerkinElmer 384-Optiplate #6007290 or EnSpire Alpha 2390 Multilabel Reader.

The negative Control and Positive Control are important to determine the range of the assay. We recommend performing all assays in duplicate.

Troubleshooting Guide

Visit bpsbioscience.com/assay-kits-faq for detailed troubleshooting instructions. For all further questions, please email support@bpsbioscience.com

References

Vasta, J.D., *et al.* 2022. *Nature Chem Biol* **18**(6): 596-604.

Nuevo-Tapiolas, C, Philips, M.R. 2022. *Front Cell Dev Biol* **10**: 10333348

Related Products

<i>Products</i>	<i>Catalog #</i>	<i>Size</i>
KRAS(G12C) Nucleotide Exchange Assay Kit	79859	384 reactions
KRAS(G12D) Nucleotide Exchange Assay Kit	78355	384 reactions
KRAS(G12V) Nucleotide Exchange Assay Kit	78519	384 reactions
KRAS(G12C) Coupled Nucleotide Exchange Assay Kit	78565	384 reactions