Description

The KRAS(G12D) Coupled Nucleotide Exchange Assay Kit is designed for screening and profiling of KRAS(G12D) antagonists/inhibitors by monitoring the binding of an effector protein such as the Ras binding domain of Raf1, (RBD-cRaf) to KRAS(G12D). The KRAS(G12D) Coupled Nucleotide Exchange Assay Kit comes in a convenient 384-well format, with enough purified recombinant **GDP-loaded KRAS(G12D) Isoform A**, GTP, exchange factor SOS1 (amino acids 564-1049), effector protein RBD-cRAF (amino acids 50-140), 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(G12D) 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(G12D) 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(G12D) so that GTP can occupy the nucleotide binding pocket. This results in a conformational change in KRAS(G12D) that permits its binding to RBD-cRAF. The KRAS(G12D) Coupled Nucleotide Exchange Assay Kit utilizes GST-tagged RBD-cRAF and His-tagged KRAS (G12D) to assay binding of KRAS(G12D) to RBD-cRAF in the Alpha assay. Glutathione acceptor and Ni chelate donor beads are brought into proximal range by the binding of KRAS(G12D) and RBD-cRAF, enabling the energy transfer from the donor to acceptor beads after laser excitation.

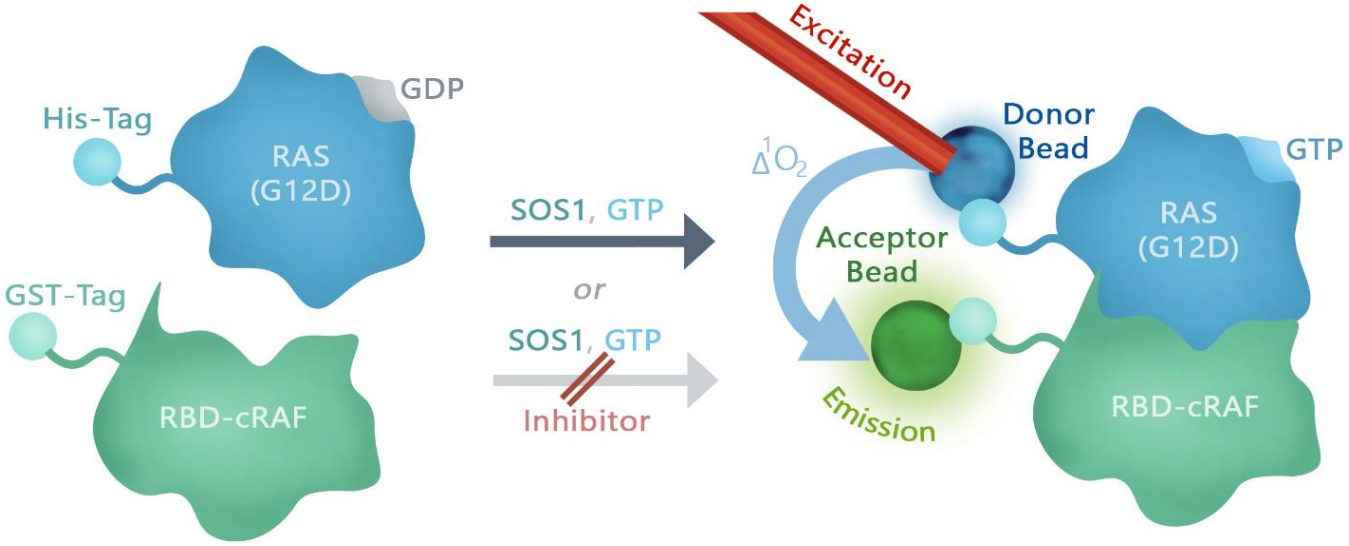

Figure 1: Illustration of the assay principle.

Background

It is well established that RAS mutations are responsible for more than 30% of human cancers. KRAS(G12D) is the most common mutation (33%) among KRAS mutant tumors. The G12D mutation favors the activated (GTP-bound) state of KRAS, amplifying signaling pathways that lead to oncogenesis. Recent studies have led to the discovery of a small molecule called MRTX1133 (Mirati) that locks KRAS conformation in the GDP-bound inactive state, thereby blocking KRAS(G12D)-mediated signaling pathway. Compounds that affect the nucleotide exchange (GDP to GTP) reaction could lead to a novel approach leading to the inhibition of tumor cell growth in KRAS(G12D) driven tumors.

Applications

Screen small molecule inhibitors or antagonists that affect KRAS(G12D) nucleotide-binding status in high throughput screening (HTS) applications.

Supplied Materials

Catalog #	Name	Amount	Storage
101312	KRAS(G12D), Isoform A, His-Tag, GDP-Loaded *	5 µg	-80°C
101573	SOS1, FLAG-Tag*	50 µg	-80°C
100519	RBD-cRAF, GST-Tag*	5 µg	-80°C
79861-2	10 mM GTP	0.5 ml	-20°C
	RBD-RAS Binding Buffer (Incomplete)	2 x 3 ml	-20°C
	0.5 M DTT	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 the no-wash alternatives to ELISA immunoassays using the 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(G12D) 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(G12D) 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 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.
- The final concentration of DMSO in the reaction should not exceed 1%.

Assay Protocol

- All samples and controls should be tested in duplicate.
- We recommend preincubating the GDP-loaded KRAS(G12D) with inhibitors if the inhibition mechanism is similar to MRTX1133; however, it is acceptable to add the GTP and SOS1 without the preincubation step.
- If the assay is going to be used more than once, aliquot remaining undiluted reagents into single-use aliquots (volumes lower than 5 µl are not recommended) depending on how many times the assay plate will be used. Store the aliquots at -80°C or as recommended for each reagent.
- This assay should include "Positive Control", "Negative Control" and "Test Inhibitor" conditions.

1. Prepare **Complete RBD-RAS Binding Buffer** by adding 6 µl of 0.5M DTT to 3 ml of RBD-RAS Binding Buffer (Incomplete). Mix well.

2. Thaw **GDP-loaded KRAS(G12D)** on ice. Briefly spin the tube containing the protein to recover the full content of the tube.

3. Dilute **GDP-loaded KRAS(G12D)** to 3 ng/µl in Complete RBD-RAS Binding Buffer (4 µl/well).

   Note: The concentration of GDP-loaded KRAS(G12D) provided may vary. Verify the concentration of the GDP-loaded KRAS(G12D) written on the tube and dilute accordingly. Prepare only the amount required for the assay. Discard any unused diluted KRAS(G12D). GDP loaded KRAS(G12D) is very sensitive to freeze/thaw cycles. Do not re-use thawed aliquots and do not re-use the diluted protein.

4. Add 4 µl/well of diluted **GDP-loaded KRAS(G12D)** to all wells.

5. Prepare the Test Inhibitor (2 µl/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.

   5.1 If the Test Inhibitor is water-soluble, prepare serial dilutions 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

   5.2 If the Test inhibitor is soluble in DMSO, prepare the test inhibitor at a concentration that is 100-fold higher than the highest desired concentration 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. The concentration of DMSO is now 5%.

Prepare serial dilutions of the Test Inhibitor at concentrations 5-fold higher than the desired final concentrations using 5% DMSO in Complete RBD-RAS Binding Buffer to keep the concentration of DMSO constant.

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).

Note: The final concentration of DMSO should not exceed 1%.

6. Add 2 µl of serial dilutions of the Test Inhibitor to the "Test Inhibitor" wells.

7. Add 2 µl of Diluent Solution to the "Positive Control" and "Negative Control" wells.

8. Briefly centrifuge the plate and incubate for 30 minutes at Room Temperature (RT).

9. Thaw **10 mM GTP** on ice.

10. Thaw **SOS1** on ice. Briefly spin the tube containing the protein to recover the full content of the tube.

11. Dilute **SOS1** in Complete RBD-RAS Binding Buffer to a concentration of 120 ng/µl (1 µl/well).

    Note: The concentration of SOS1 provided may vary. Verify the concentration of the protein written on the tube and dilute accordingly. Prepare only the amount required for the assay. Discard any unused diluted SOS1. SOS1 is very sensitive to freeze/thaw cycles. Do not re-use thawed aliquots and do not re-use the diluted protein.

12. Combine 10 mM GTP and diluted SOS1 at a 1:1 ratio (2 µl/well): N wells × (1 µl of diluted SOS1 + 1 µl of 10 mM GTP).

13. Initiate the exchange reaction by adding 2 µl of GTP/SOS1 mix to the "Test Inhibitor" and the "Positive control" wells.

14. Add 2 µl of Complete RBD-RAS Binding Buffer to the "Negative Control" wells.

15. Briefly centrifuge the plate and incubate at RT for 30 minutes.

16. Thaw **RBD-cRAF** on ice. Briefly spin the tube containing the protein to recover the full content of the tube.

17. Dilute **RBD-cRAF** with Complete RBD-RAS Binding Buffer to a concentration of 1.8 ng/µl (2 µl/ well).

    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. Prepare only the amount required for the assay. Discard unused diluted RBD-cRAF. RBD-cRAF is very sensitive to freeze/thaw cycles. Do not re-use thawed aliquots and do not re-use the diluted protein.

18. At the end of the 30-minute incubation with SOS1/GTP, add 2 µl of the diluted **RBD-cRAF** to all wells.

19. Briefly centrifuge the plate and incubate at RT for 30 minutes.

20. Dilute **3X Immuno Buffer** 3-fold with deionized water to prepare 1X Immuno Buffer.

21. 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 (20 µl of mix/well).

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

22. Add 20 µl of acceptor/donor beads mixture to all the wells.

23. Incubate 30 min at RT.

24. Read Alpha-counts using a compatible plate reader (PerkinElmer).

Component	Negative Control	Positive Control	Test Inhibitor
Diluted GDP-loaded KRAS(G12D) (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)/Diluted SOS1 mixture	-	2 µl	2 µl
Complete RBD-RAS Binding Buffer	2 µl	-	-
Centrifuge and incubate	30 minutes at room temperature		
Diluted RBD-cRAF (1.8 ng/µl)	2 µl	2 µl	2 µl
Centrifuge and incubate	30 minutes at room temperature		
Total	**10 µl**	**10 µl**	**10 µl**

Example Results

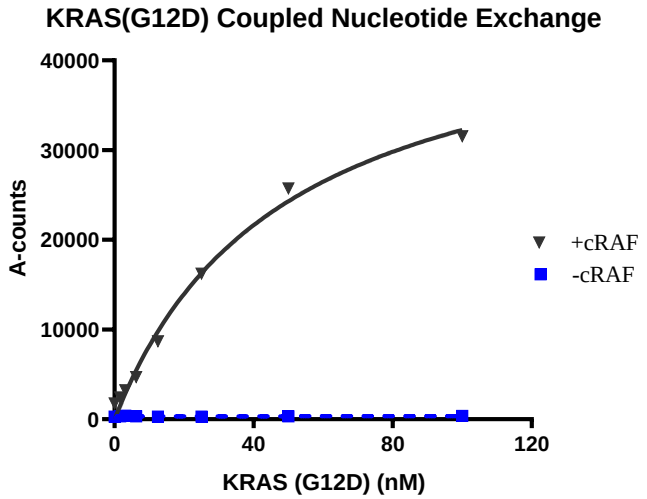

Figure 1: Nucleotide exchange of KRAS (G12D).
The nucleotide exchange of KRAS(G12D) was evaluated in the presence or absence of cRAF.

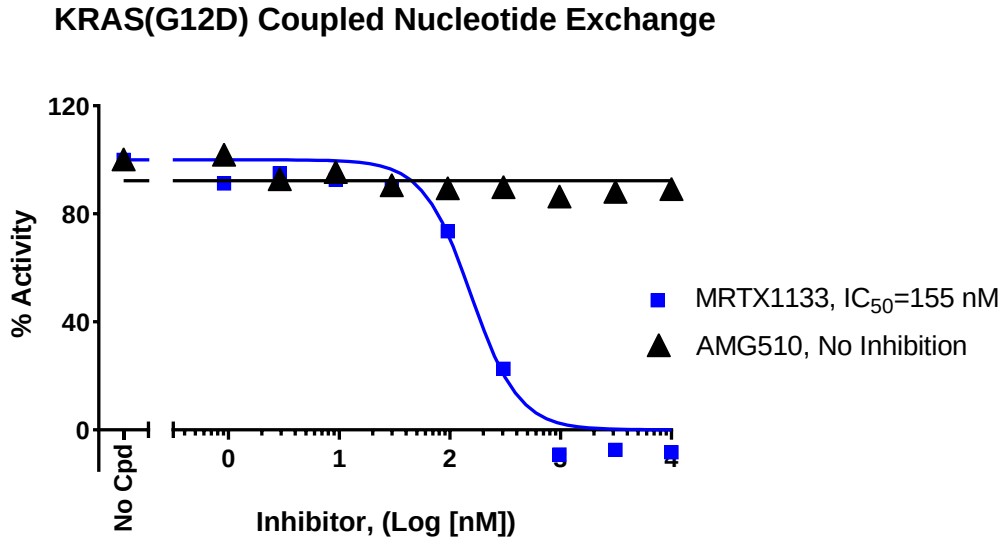

Figure 2: Effect of MRTX1133 and AMG510 on the nucleotide exchange of KRAS(G12D).
Inhibition of the nucleotide exchange of KRAS(G12D) was evaluated in the presence of increasing concentrations of KRAS(G12D) and KRAS (G12C)-specific inhibitors.

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 Mutilabel Reader.

Troubleshooting Guide

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

References

Wang, X., *et al.,* 2022 *J Med Chem* 65: 3123-33.
Hillig, R.C., *et al., 2019 PNAS USA* 116 (7): 2551-2560

Related Products

Products	*Catalog #*	*Size*
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