Description

The KRAS(G12D) Nucleotide Exchange Assay Kit is a fluorogenic homogeneous assay designed for the screening and profiling of KRAS (Kirsten rat sarcoma) (G12D) antagonists/inhibitors by using BODIPY®-GDP to monitor the KRAS(G12D) GDP or GTP binding status. The KRAS(G12D) Nucleotide Exchange Assay Kit comes in a convenient 96-well format, with enough purified recombinant KRAS(G12D) isoform A labeled with BODIPY®-GDP, GTP, assay buffer and additives for 100 enzyme reactions. Two different protocols can be used with this kit, allowing greater experimental flexibility. It can used to either titrate the inhibitor at a fixed GTP concentration, or to titrate GTP in the presence of a fixed inhibitor concentration.

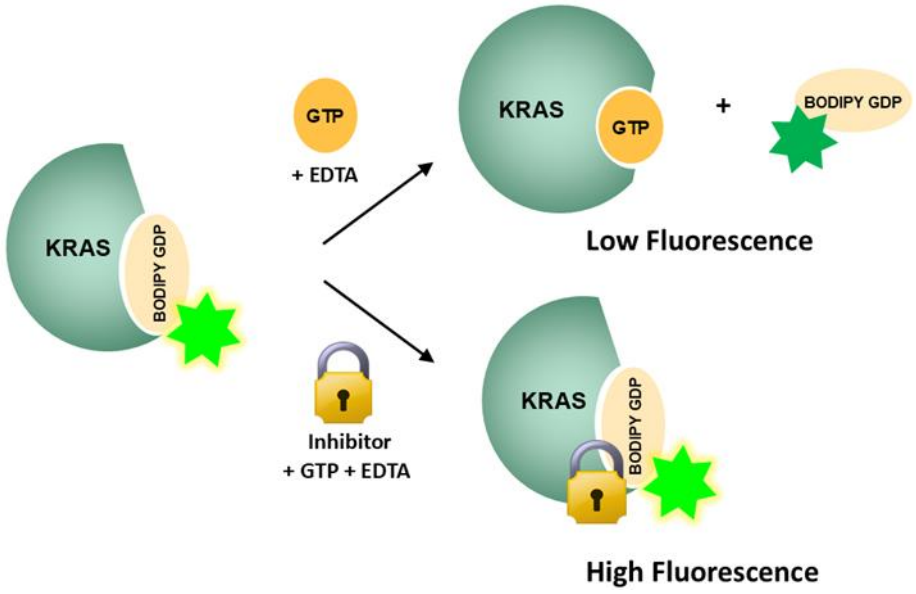

Figure 1: Schematic representation of the mechanism of the KRAS(G12D) Nucleotide Exchange Assay Kit.
KRAS is activated upon binding GTP and undergoes a conformational change. KRAS then returns to a GDP-bound inactive state following the hydrolysis of GTP to GDP. In this assay, KRAS is pre-loaded with fluorescent BODIPY-GDP and therefore is inactive. Adding GTP in the presence of EDTA displaces BODIPY-GDP because the affinity of KRAS for GTP is greater than its affinity for GDP. The fluorescence intensity decreases as the BODIPY-GDP is displaced, since free BODIPY-GPD has low fluorescence versus when protein-bound. Several KRAS inhibitors lock KRAS in the (inactive) GDP-bound conformation and prevent GDP/GTP exchange. In this scenario the fluorescence intensity remains high and corelates with drug concentration as more BODIPY--GDP stays bound to KRAS.

Background

It is well established that RAS mutations are responsible for more than 30% of human cancers. KRAS(G12D) is one of the KRAS mutations that is found frequently in pancreatic and colon cancers. Recent studies have led to the discovery of a small molecule called MRTX-1133 that locks KRAS conformation in the inactive GDP-bound state, thereby blocking the KRAS(G12D)-mediated signaling pathway. Compounds that affect the nucleotide exchange (GDP to GTP) reaction in KRAS may inhibit tumor cell growth in KRAS(G12D)-driven tumors.

BODIPY FL-GDP is a mixed isomer in which the fluorophore has been attached to the 2' or 3' position of the ribose ring via a linker. It is a green-fluorescent dye with similar excitation and emission to fluorescein or Alexa Fluor™ 488, characterized by a high extinction coefficient and high quantum yield and is relatively insensitive to pH changes. The dye has an excited-state lifetime of 5 nanoseconds or longer. It is a commonly used dye to fluorescently label GTPases and determine their GTP/GDP status.

Applications

Study enzyme kinetics and screen small molecular inhibitors of KRAS(G12D) for drug discovery and High Throughput Screening (HTS) applications.

Supplied Materials

Catalog #	Name	Amount	Storage
100887	KRAS (G12D), Isoform A, BODIPY®-GDP Loaded, His-Tag*	60 µg x 2	-80°C
79861-1	Guanosine 5'-triphosphate (GTP) (10 mM)	100 µl	-20°C
79862	2x KRAS Nucleotide Exchange Buffer (Assay Buffer)	5 ml	-20°C
82735	0.5 M DTT	200 µl	-20°C
82763	0.5 M EDTA	100 µl	Room Temp.
79685	96-well black microplate	1	Room Temp.
	Plate Sealing film		

**The molecular weight of the protein is 23 kDa. The volume provided is ≥520 µl/tube (x2 tubes), which is sufficient for 10 µl/well as described in the protocol.*

Materials Required but Not Supplied

- Fluorescent microplate reader capable of reading λex/em=470 nm/525 nm
- Adjustable micropipettor and sterile tips

Stability

This assay kit will perform optimally for up to **6 months** from date of receipt when the materials are stored as directed.

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.

Contraindications

- The final concentration of DMSO in the assay should not exceed 1%.
- Compounds that are fluorescent may interfere with the results, depending on their spectral excitation and emission properties.
- It is recommended that the compound alone is tested to determine any potential interference of the compound on the assay results.

Assay Protocol

- All samples should be run in duplicate while controls should be performed in quadruplicate.
- For protocol A the assay should include "Positive Control", "Negative Control" and "Test Inhibitor" conditions.
- For protocol B, where GTP is being titrated, both the "Positive Control" and "Test Inhibitor" conditions should be tested in the presence of the several GTP dilutions.

- We recommend maintaining the diluted protein on ice during use.
- For detailed information on protein handling please refer to Protein FAQs (bpsbioscience.com).
- We recommend using MRTX-1133 (#82718) as internal control. If not running a dose response curve for the control inhibitor, we recommend running the control inhibitor at 0.1x, 1x and 10X the IC_{50} value shown in the validation data below.
- For instructions on how to prepare reagent dilutions please refer to Serial Dilution Protocol (bpsbioscience.com).

A. Inhibitor titration at a fixed GTP concentration

1. Thaw **2x KRAS Assay Buffer**, **0.5 M DTT**, and **BODIPY-GDP loaded KRAS(G12D)** on ice. Briefly spin the tube containing the protein to recover its full contents.

   Note: BODIPY-GDP loaded KRAS(G12D) is sensitive to freeze/thaw cycles. Avoid multiple freeze/thaw cycles. Do not freeze and re-use the diluted protein.

2. Prepare 1x KRAS Assay Buffer containing 1 mM DTT by diluting 2x KRAS Assay Buffer 2-fold and adding DTT.

   Example: For 4 ml of 1x KRAS Assay Buffer, mix 2 ml of 2x KRAS Buffer with 8 µl of 0.5 M DTT and 1,992 µl of distilled water.

3. Prepare a **Master Mix** (35 µl/ well): N wells x (10 µl of BODIPY-GDP loaded KRAS(G12D) + 25 µl of 1x KRAS Assay Buffer containing 1 mM DTT).

4. Add 35 µl of the Master Mix to all wells.

5. Prepare the Test Inhibitor (5 µl/well): For the titration, prepare serial dilutions at concentrations 10-fold higher than the desired final concentrations. The final volume of the reaction is 50 µl.

   5.1 If the Test Inhibitor is water-soluble, prepare serial dilutions in 1x KRAS Assay Buffer containing 1 mM DTT, 10-fold more concentrated than the desired final concentrations.

   Note: We recommend preparing 3-fold increments serial dilutions.

   For positive control, use 1x KRAS Assay Buffer containing 1 mM DTT (Diluent Solution).

   OR

   5.2 If the Test Inhibitor is soluble in DMSO, prepare the Test Inhibitor at 100-fold the highest desired concentration in DMSO. Dilute the inhibitor 10-fold in 1x KRAS Assay Buffer containing 1 mM DTT to prepare the highest concentration of the 10-fold concentrated intermediate solution.

   Prepare serial dilutions of the Test Inhibitor at 10-fold the desired final concentrations using 10% DMSO prepared in 1x KRAS Assay Buffer containing 1 mM DTT (Diluent Solution) to keep the concentration of DMSO constant.

Note: We recommend preparing 3-fold increments serial dilutions.

For Positive control, prepare 10% DMSO in 1x KRAS Assay Buffer containing 1 mM DTT (vol/vol) so that all wells contain the same amount of DMSO (Diluent Solution).

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

6. Add 5 µl of the diluted inhibitor to the "Test Inhibitor" wells.
7. Add 5 µl of Diluent Solution to the "Positive Control" and "Negative Control" wells.
8. Centrifuge the plate to ensure all the components are mixed well and incubate the plate for 2 hours at Room Temperature (RT).
9. Thaw **10 mM GTP** and **0.5 M EDTA**.
10. Prepare 30 µM GTP in distilled water (5 µl/well) by performing a serial dilution.

    Example: Dilute 10 mM GTP 10-fold with distilled water to prepare 1 mM GTP. Then, dilute the 1 mM GTP solution 33-fold to make a 30 µM solution.
11. Prepare 25 mM EDTA by diluting 0.5 M EDTA 20-fold with distilled water (5 µl/well).
12. Mix diluted GTP (30 µM) and EDTA (25 mM) at a 1:1 ratio [(5 µl +5 µl)/well].
13. After the 2-hour incubation, initiate the reaction by adding 10 µl of the GTP/EDTA Mix to the "Positive Control" and "Test Inhibitor" wells.
14. Add 10 µl of distilled water to the "Negative Control" wells.
15. Read the fluorescence at λex470 nm/λem525 nm in a microtiter-plate reader in kinetic mode. An end point readout can be done in 20-25 minutes.

Component	**Positive Control (Low Fluorescence)**	**Test Inhibitor**	**Negative Control (High Fluorescence)**
Master Mix	35 µl	35 µl	35 µl
Test Inhibitor	-	5 µl	-
Diluent Solution	5 µl	-	5 µl
Incubate 2 hours at RT			
GTP/EDTA Mix	10 µl	10 µl	-
Distilled water			10 µl
Total	**50 µl**	**50 µl**	**50 µl**

Example of Assay Results:

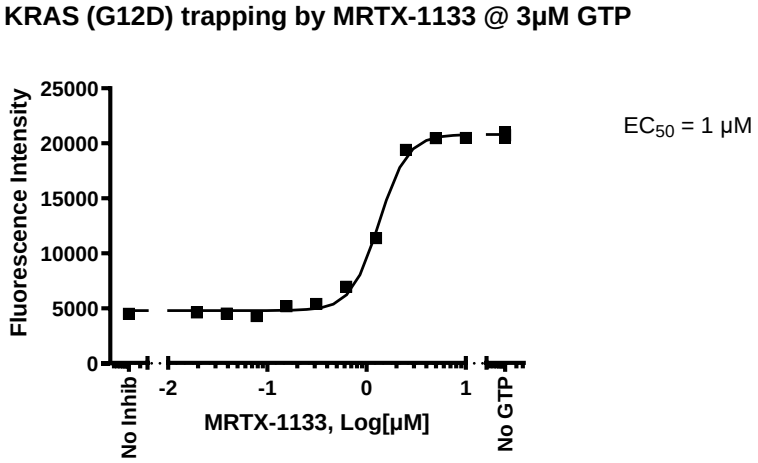

Figure 2. KRAS(G12D) trapping in the BODIPY-GDP-bound state by MRTX-1133.
KRAS (G12D) trapping was measured in the presence of a fixed GTP concentration (3 µM) and increasing concentrations of MRTX-1133. In the absence of GTP, BODIPY-GDP remains bound to KRAS and has high fluorescence intensity. Adding GTP in the presence of EDTA displaces BODIPY-GDP from KRAS decreasing its fluorescence intensity. The presence of MRTX-1133 inhibitor in the reaction locks KRAS in the (inactive) BODIPY-GDP-bound conformation and abrogates the BODIPY-GDP release from KRAS in a dose-dependent fashion.

B. GTP titration at a fixed inhibitor/antagonist concentration

1. Thaw **2x KRAS Assay Buffer**, **0.5 M DTT**, and **BODIPY-GDP loaded KRAS(G12D)** on ice. Briefly spin the tube containing the protein to recover the full contents.

   Note: BODIPY-GDP loaded KRAS(G12D) is sensitive to freeze/thaw cycles. Avoid multiple freeze/thaw cycles. Do not freeze and re-use the diluted protein.

2. Prepare 1x KRAS Assay Buffer containing 1 mM DTT by diluting 2x KRAS Assay Buffer 2-fold and add DTT.

   Example: to prepare 4 ml of 1x KRAS Assay Buffer mix 2 ml of stock 2x KRAS Assay Buffer with 8 µl of 0.5 M DTT and 1,992 µl of distilled water.

3. Prepare a Master Mix (35 µl/well): N wells X (10 µl of BODIPY-GDP loaded KRAS(G12D) + 25 µl of 1x KRAS Assay Buffer containing 1 mM DTT).

4. Add 35 µl of the Master Mix to all wells.

5. Prepare the Test Inhibitor (5 µl/well) at a concentration 10-fold higher than the desired final concentration. The final volume of the reaction will be 50 µl.

   Note: In this protocol, the inhibitor is used at a fixed concentration.

5.1 If the Test Inhibitor is water-soluble, prepare a 10-fold concentrated compound solution in 1x KRAS Assay Buffer containing 1 mM DTT.

For Positive Control, use 1x KRAS Assay Buffer containing 1 mM DTT (Diluent Solution).

OR

5.2 If the Test Inhibitor is soluble in DMSO, dissolve the Test Inhibitor at 100-fold the desired final concentration in DMSO. Dilute the inhibitor 10-fold in 1x KRAS Assay Buffer containing 1 mM DTT to prepare the 10-fold concentrated intermediate solution. The concentration of DMSO in the intermediate solution is 10%.

For Positive Control, prepare 10% DMSO in 1x KRAS Assay Buffer containing 1 mM DTT (vol/vol) so that all wells contain the same amount of DMSO (Diluent Solution).

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

6. Add 5 µl of Test Inhibitor to each well labeled "Test Inhibitor".

7. Add 5 µl of Diluent Solution to the "Positive Control".

8. Centrifuge the plate to ensure all the components are mixed well and incubate the plate for 2 hours at RT.

9. Thaw the **10 mM GTP** and **0.5 M EDTA**.

10. Make serial dilutions of GTP from 0 mM to 1 mM in distilled water (5 µl/well). We recommend preparing 3-fold increments serial dilutions.

    Note: At this point GTP is 10-fold more concentrated than the desired final concentrations. The final GTP concentrations in the reaction will be 0 to 100 µM.

11. Prepare 25 mM EDTA by diluting 0.5 M EDTA 20-fold with distilled water (5 µl/well).

12. After the 2-hour incubation, add 5 µl of the serially diluted GTP to all the wells.

13. Initiate the reaction by adding 5 µl of 25 mM EDTA to all the wells.

Component	Positive Control	Test Inhibitor
Master Mix	35 µl	35 µl
Test Inhibitor (at a fixed concentration)	-	5 µl
Diluent Solution	5 µl	-
Incubate 2 hours at RT		
Diluted GTP (titration)	5 µl	5 µl
Diluted EDTA (25 mM)	5 µl	5 µl
Total	**50 µl**	**50 µl**

14. Centrifuge the plate to ensure that all the components are mixed well.

15. Read the fluorescence at λex470 nm/λem525 nm in a microtiter-plate reader in kinetic mode. An end point readout can be done in 20-25 minutes.

Example of Assay Results:

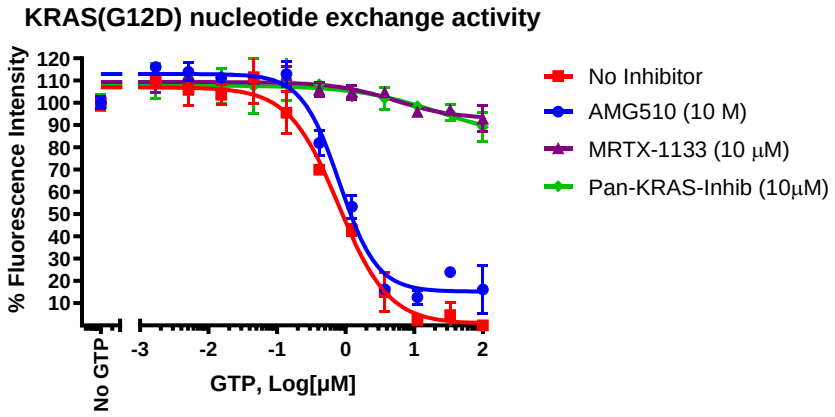

Figure 3: Effect of various KRAS inhibitors on locking the KRAS(G12D) mutant in the BODIPY-GDP-bound state and inhibiting GDP-GTP exchange reaction.
KRAS (G12D) nucleotide exchange activity was measured in the presence of increasing GTP concentrations, with or without AMG510 (10 µM), MRTX-1133 (10 µM) and PAN-KRAS-Inhibitor (10 µM). Increasing the GTP concentration in the reaction improves BODIPY-GDP displacement from KRAS(G12D), which is manifested by a decrease in its fluorescence intensity. Adding MRTX-1133 and PAN-KRAS-Inhibitor but not AMG510 inhibitor to the reaction traps KRAS(G12D) in the inactive GDP-bound state abrogating the nucleotide exchange process.

Results are representative.

Troubleshooting Guide
Visit bpsbioscience.com/assay-kits-faq for detailed troubleshooting instructions. For lot-specific information and all other questions, please visit https://bpsbioscience.com/contact.

References

Ostrem J.M., *et al*., 2013. *Nature* 503: 548-551.
Patricelli M.P., *et al*., 2016. *Cancer Discover*. 6: 316-329.

Related Products

Products	*Catalog #*	*Size*
KRAS(G12V) Nucleotide Exchange Assay Kit	78519	384 reactions
KRAS(G12D) Nucleotide Exchange Assay Kit	78355	384 reactions
KRAS(G12C) Coupled Nucleotide Exchange Assay Kit	78004	384 reactions
KRAS(G12C) Isoform A Coupled Nucleotide Exchange TR-FRET Assay Kit	82709	384 reactions
KRAS (G12C), Isoform A, His-Tag, BODIPY-GDP Loaded	100537	20 µg
KRAS (G12C), Isoform A, His-Tag, GDP-Loaded	100640	4 x 50 µg

Version 051225