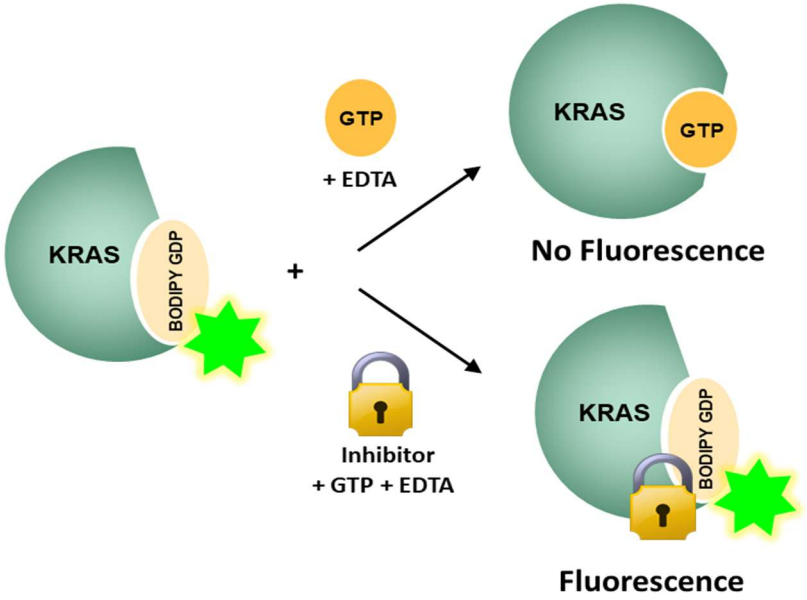
Description

The KRAS(G12C) Nucleotide Exchange Assay is a homogeneous assay designed for the screening and profiling of KRAS(G12C) antagonists/inhibitors by using BODIPY®-GDP to monitor the GDP or GTP binding status. The KRAS(G12C) Nucleotide Exchange Assay Kit comes in a convenient 384-well format, with enough purified recombinant KRAS(G12C) labeled with BODIPY®-GDP, GTP, assay buffer and additives for 400 enzyme reactions. The kit can be used with two different protocols for greater flexibility, either titrating the inhibitor at a fixed GTP concentration or titrating the GTP at a fixed inhibitor concentration.

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.



Assay Principle, Illustration: KRAS is activated upon binding GTP, when it 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 KRAS affinity for GTP is greater than its affinity for GDP. The fluorescence intensity decreases as the BODIPY-GDP is displaced. Several KRAS inhibitors lock KRAS in the (inactive) GDP-bound conformation and prevent GDP/GTP exchange. In this scenario the fluorescence intensity increases with drug concentration as more BODIPY--GDP remains bound to KRAS.

Background

It is well established that RAS mutations are responsible for more than 30% of human cancers. KRAS(G12C) is the most frequent mutation, found in lung and colon cancers. Recent studies uncovered a small molecule called AMG510 (Amgen) that inhibits the KRAS(G12C)-mediated signaling pathway by locking the KRAS conformation in the GDP-bound state. Compounds that block the nucleotide exchange (GDP to GTP) reaction in KRAS could lead to the inhibition of tumor cell proliferation in KRAS(G12C)-driven tumors.

Mutations

G12C

Applications

Study enzyme kinetics and screen small molecular inhibitors of KRAS(G12C) for drug discovery and High Throughput (HTS) applications.

Supplied Materials

Catalog #	Name	Amount	Storage
100537	KRAS (G12C), Isoform A, His-Tag, BODIPY®-GDP Loaded, 5 µM	60 µg X 4	-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
	DTT, 0.5 M	200 µl	-20°C
	EDTA, 0.5 M	100 µl	Room Temp.
79961	384-well plate, Black	1	Room Temp.

Note: The molecular weight of the protein is 23 kDa. The volume provided is ≥520 µl/tube (x4 tubes), which is sufficient for 5 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.

Assay Protocols

All samples and controls should be tested in duplicate.

A. GTP titration at a fixed inhibitor/antagonist concentration

1. Thaw 2x KRAS Assay buffer, 0.5 M DTT, and BODIPY-GDP loaded KRAS(G12C) on ice. Briefly spin the tube containing the protein to recover the full contents.

   Note: BODIPY-GDP loaded KRAS(G12C) 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.
   Example: To make 4 ml of 1X KRAS Assay Buffer, mix 2 ml of stock 2X KRAS Assay Buffer + 8 µl of DTT (0.5 M) + 1,992 µl of distilled water.

3. Prepare the Master Mix (17.5 µl/well): N wells X (5 µl of BODIPY-GDP loaded KRAS(G12C) + 12.5 µl of 1X KRAS buffer containing DTT as prepared above).

4. Add 17.5 µl of the Master Mix to all wells.

5. Prepare the Test Inhibitor (2.5 µl/well) at a concentration 10-fold higher than the desired final concentration. The final volume of the reaction will be 25 µl.

   Note that in this protocol, the inhibitor is used at a fixed concentration.

   Without DMSO:

   a. If the Test Inhibitor is water-soluble, prepare a 10-fold concentrated compound solution in 1x KRAS Buffer containing DTT.
   b. For the positive control, use 1x KRAS Buffer containing DTT but no inhibitor (Diluent Solution).

   With DMSO:

   a. If the Test Inhibitor is soluble in DMSO, dissolve the Test Inhibitor at 100-fold the desired final concentration in DMSO.
   b. Dilute the inhibitor 10-fold in 1x KRAS Assay Buffer (containing DTT) to prepare the 10-fold concentrated intermediate solution.

      Example: To test an inhibitor at a concentration of 50 µM, prepare a 5 mM inhibitor solution then add 10 µl of the 5 mM solution to 90 µl of KRAS Assay Buffer containing DTT to make a 500 µM intermediate solution. The concentration of DMSO in the intermediate solution is 10%.
   c. For the Positive control, prepare 10% DMSO in 1x KRAS Assay Buffer containing DTT (vol/vol) so that all wells contain the same amount of DMSO (Diluent Solution).

   Caution: Do not exceed 10% DMSO in the 10-fold intermediate solution. The final concentration of DMSO in the assay should not exceed 1%.

6. Add 2.5 µl of Test Inhibitor to each well labeled “Test Inhibitor”.

7. Add 2.5 µl of Diluent Solution to the control.

Component	Positive Control	Test Inhibitor
Master Mix	17.5 µl	17.5 µl
Test Inhibitor	-	2.5 µl
Diluent Solution (no inhibitor)	2.5 µl	-
Total	20 µl	20 µl

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

9. Thaw the 10 mM GTP and 0.5 M EDTA. Make serial dilutions of GTP in distilled water 10-fold more concentrated than the desired final concentrations, from 0 mM to 1 mM.

   Note: These are 10-fold concentrations, so the final GTP concentrations in the reaction will be 0 to 100 µM. We recommend preparing 3-fold increments serial dilutions.

10. Prepare 25 mM EDTA by diluting 0.5M EDTA in distilled water.
    Example: Mix 50 µl of 0.5M EDTA with 950 µl of distilled water to make 1 ml of 25 mM EDTA.

11. After the 2-hour incubation, add 2.5 µl of the serially diluted GTP to all the wells.

12. Initiate the reaction by adding 2.5 µl of 25 mM EDTA to all the wells.

    The volume of the reaction is now 25 µl.

13. Centrifuge the plate to ensure that all the components are mixed well and incubate the plate for 1 hour at room temperature.

14. Read the Fluorescence at λex470nm/em525nm.

Example of Assay Results:

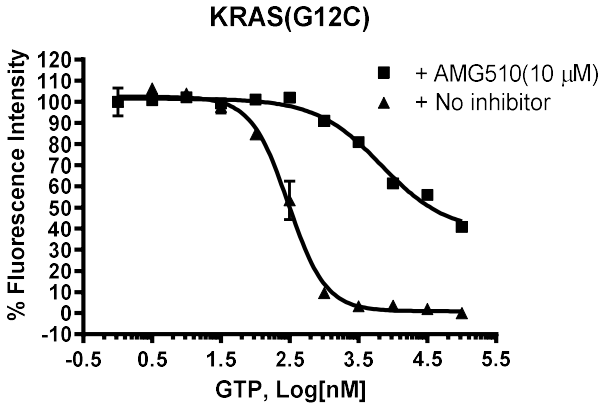


Figure 1: EDTA-mediated GDP-GTP exchange reaction of KRAS(G12C).
The assay was performed following the KRAS(G12C) Nucleotide Exchange Assay Kit protocol described above, in the presence of increasing GTP concentrations, with or without AMG510 (10 µM). Adding GTP and EDTA pushes the GDP-GTP exchange, resulting in lower ratios of the fluorescent KRAS-BODIPY-GDP form as the concentration of GTP increases. Inhibitor AMG510 blocks the GDP/GTP conversion in a dose-dependent fashion.

B. Inhibitor titration at a fixed GTP concentration

1. Thaw 2x KRAS Assay Buffer, 0.5 M DTT, and BODIPY-GDP loaded KRAS(G12C) on ice. Briefly spin the tube containing the protein to recover its full contents.

   Note: BODIPY-GDP loaded KRAS(G12C) 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.

   Example: For 4 ml of 1X KRAS buffer, mix 2 ml of 2X KRAS buffer + 8 µl of DTT (0.5 M) + 1,992 µl of distilled water.

3. Prepare the Master Mix (17.5 µl): N wells X (5 µl of BODIPY-GDP loaded KRAS(G12C) + 12.5 µl of 1X KRAS buffer prepared above).

4. Add 17.5 µl of the Master Mix to all wells.

5. Prepare the Test Inhibitor (2.5 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 25 µl.

   Without DMSO:

   a. If the Test Inhibitor is water-soluble, prepare serial dilutions in 1x KRAS Assay Buffer (containing DTT), 10-fold more concentrated than the desired final concentrations.
   Note: We recommend preparing 3-fold increments serial dilutions.

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

   With DMSO:

   a. If the Test Inhibitor is soluble in DMSO, prepare the Test Inhibitor at 100-fold the highest desired concentration in DMSO.

   b. Dilute the inhibitor 10-fold in 1x KRAS Assay Buffer (containing DTT) to prepare the highest concentration of the 10-fold concentrated intermediate solution.

      Example: To test at a maximum concentration of 50 µM, prepare a 500 µM intermediate solution by adding 10 µl of 5 mM inhibitor solution to 90 µl of KRAS Assay Buffer. The concentration of DMSO is now 10%.

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

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

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

   ⚠ *Caution: Do not exceed 10% DMSO in the 10-fold intermediate solution.*

6. Add 2.5 µl of the diluted inhibitor to the wells and centrifuge the plate to ensure all the components are mixed well.

7. Add 2.5 µl of diluent solution without inhibitor in the control.

Component	Positive Control	Test Inhibitor
Master Mix	17.5 µl	17.5 µl
Test Inhibitor	-	2.5 µl
Diluent Solution (no inhibitor)	2.5 µl	-
Total	20 µl	20 µl

8. Incubate the plate for 2 hours at room temperature.

9. Thaw 10 mM GTP and 0.5 M EDTA. Prepare 30 µM GTP in distilled water.
   Example: Dilute 10 mM GTP 10-fold in water (10 µl of GTP (10 mM) + 90 µl of water) to prepare 1 mM GTP. Dilute it 33-fold to make a 30 µM solution.

10. Dilute EDTA (0.5 M) in water to prepare 25 mM EDTA,
    Example: To prepare 1 ml of 25 mM EDTA, mix 50 µl of EDTA (0.5 M) with 950 µl of distilled water.

11. Mix diluted GTP (30 µM) and EDTA (25 mM) at a 1:1 ratio.

12. After the 2-hour incubation, initiate the reaction by adding 5 µl of the GTP/EDTA solution to all the wells.

13. Incubate for 1-hour at room temperature.

14. After 1-hour incubation, read the Fluorescence at λex470nm/λem525nm.

Example of Assay Results:

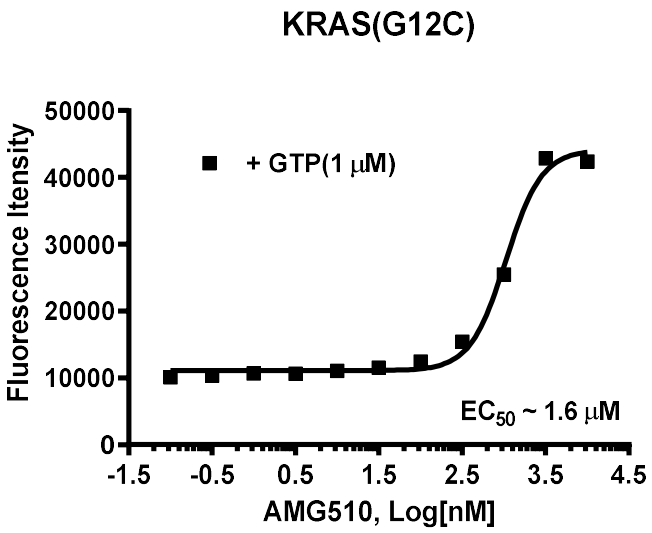


Figure 2: EDTA-mediated GDP-GTP exchange reaction of KRAS(G12C). The assay was performed following the KRAS(G12C) Nucleotide Exchange Assay Kit protocol described above, in the presence of a fixed GTP concentration and increasing concentrations of AMG510. Signal increases in the presence of the inhibitor as increasing amounts of KRAS (G12C) are locked in the BODIPY®-GDP-bound conformation.

Results are representative. For lot-specific information, please contact BPS Bioscience, Inc. at support@bpsbioscience.com

Troubleshooting Guide

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

References

Ostrem JM, *et al*., 2013. *Nature* **503**: 548-551.
Patricelli MP, *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, His-Tag, BODIPY-GDP Loaded	100537	20 µg
KRAS (G12C), Isoform A, His-Tag, GDP-Loaded	100640	4 x 50 µg
KRAS (G12C), Isoform A, His-Tag (*E. coli* derived)	100413	100 µg
KRAS (G13D), Isoform B, His-Tag	100479	100 µg
KRAS (G12R), Isoform B, His-Tag (Sf9-derived)	100825	100 µg
KRAS, Isoform B, His-Tag	11308	100 µg