KRAS nucleotide exchange assays for inhibitor screening and profiling

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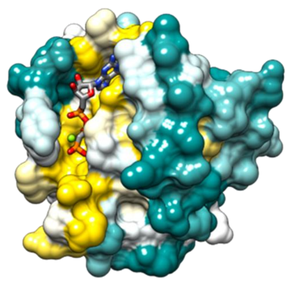
Background



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RAS (Rat Sarcoma Virus) is a family of small GTPases involved in cell signal transduction. Like most small GTPases, KRAS binds to GDP in its inactive form and to GTP in its active form. Nucleotide exchange is facilitated by Guanylate exchange factors (GEFs) such as SOS (Son of Sevenless) and gp120 GAP (GTPase activating proteins). While gp120GAP catalyzes the hydrolysis of GTP to GDP in Ras/GTP, thereby inactivating RAS, SOS promotes the exchange of GDP for GTP and activates RAS.

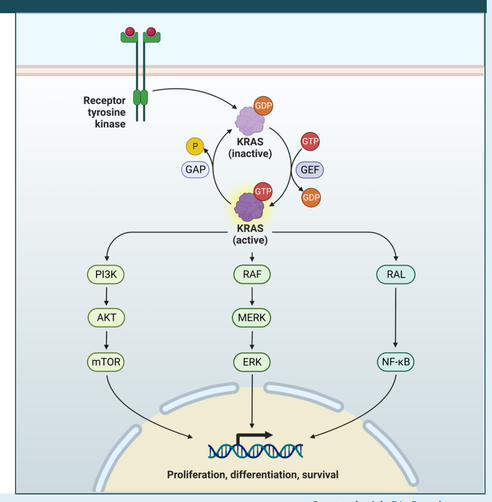
Comprising three members KRAS, NRAS and HRAS, it is the most frequently mutated family of oncogenes in human cancer. Indeed, RAS mutations are responsible for more than 30% of tumors. KRAS is the predominant mutant form (85%), whereas NRAS and HRAS are infrequent (11% and 4%, respectively).

More than 70% of RAS mutations occur at the G12, G13 or Q61 positions of the RAS protein accounting for most pancreatic, colorectal and non-small cell lung cancers. Up to about 10 years ago, KRAS was still considered “undruggable”. Fortunately, Ostrem et al. [PMID: 24256730] opened the door to allele-specific inhibition through covalent targeting of the mutant-specific free cysteine residue.

The G12C mutation favors the activated (GTP-bound) state of KRAS, amplifying signaling pathways that lead to oncogenesis. KRAS(G12C) is found in colon and lung cancer and represents an attractive therapeutic target. Two inhibitors have now been developed to block KRAS(G12C)-mediated signaling pathway by locking KRAS in its inactive form.

The advancement of these two KRAS(G12C) inhibitors has spurred new efforts in the field. Considering the frequency of RAS mutations in human cancers and the paucity of options for many patients with RAS-induced cancer, there is a large market for pharmaceutical companies to meet a pressing need.

Drug discovery and development projects require reliable assays to screen and evaluate new small molecules that potentially inhibit RAS isoforms. **We describe the development of two types of assays for high-throughput screening applications and titration of candidate compounds. One assay is based on AlphaScreen® technology, while the other takes advantage of BODIPY®-GDP in fluorophore GDP/GTP exchange assay kits.**



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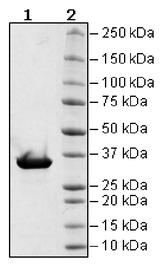
Affinity Purified Proteins

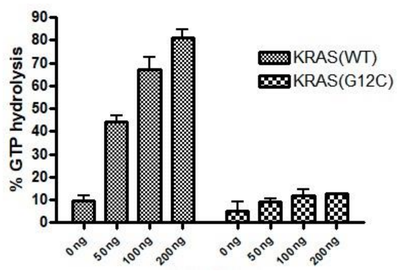
KRAS	Isoform	Nucleotide load	Catalog number
KRAS Wild-type	B		11308
KRAS Wild-type	B	GDP	101522
KRAS Wild-type	B	BODIPY-GDP	100886
KRAS-G12C	A		100413
KRAS-G12C	A	GDP	100640
KRAS-G12C	A	GppNHp	100641
KRAS-G12C	A	BODIPY-GDP	100537
KRAS-G12C	B		100824
KRAS-G12D	A		100623
KRAS-G12D	A	GDP	101312

KRAS	Isoform	Nucleotide load	Catalog number
KRAS-G12D	A	GppNHp	101481
KRAS-G12D	A	BODIPY-GDP	100887
KRAS-G12R	A		100841
KRAS-G12R	B		100825
KRAS-G12V	B		100480
KRAS-G12V	B	GDP	101355
KRAS-G12V	B	GppNHp	101359
KRAS-G12V	B	BODIPY-GDP	101504
KRAS-G13D	B		100479

The quality of a KRAS biochemical assay depends on the purified proteins. Wild-type and mutant KRAS were all constructed with a His-Tag to allow for affinity purification. Purified proteins are loaded with GDP, non-hydrolyzable GTP analog GppNHp, or BODIPY-GDP, depending on the application. NRAS(Q61H), NRAS(Q61L) and NRAS(Q61K) are also available as purified proteins.



gp120GAP (aa 714-1047)



Left panel: gp120GAP purity was assessed by 4-20% SDS-PAGE electrophoresis followed by Coomassie Staining. Right panel: GTPase activity of KRAS-WT (50 ng) and KRAS-G12C (100 ng) was tested with increasing concentrations of p120GAP and 2 µM GTP using GTPase-Glo reagents (Promega) at room temperature for 80 min.

Regulator	Tag	Label	amino acids	Catalog number
gp120GAP	His		714-1047	100518
SOS1	Flag, Avi™		594-1049	100752
SOS1	Flag, Avi™	Biotin	594-1049	100753

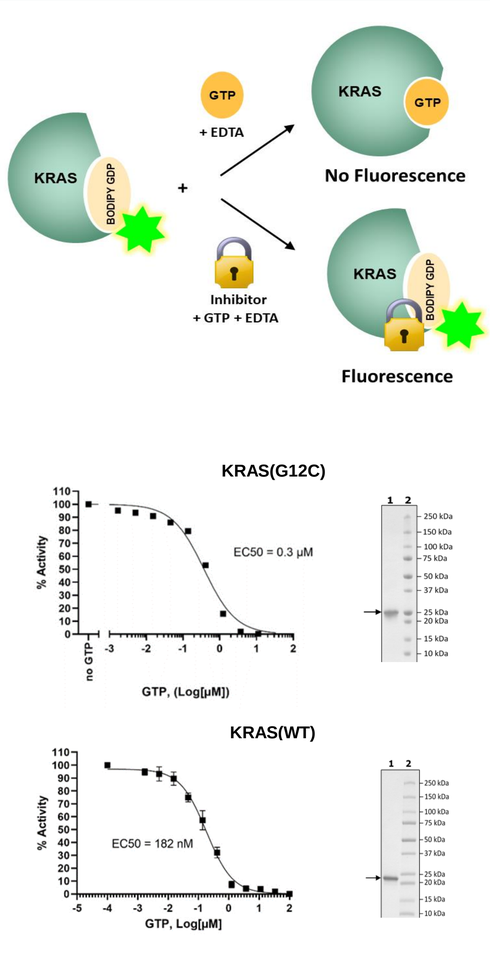
KRAS regulator gp120GAP protein (Ras GTPase-activating protein 1), encompassing amino acids 714-1047, contains the RAS-GAP functional domain and an N-terminal His-Tag. It is used to deactivate KRAS by promoting exchange of GTP for GDP. On the other hand, SOS1 is used to activate KRAS by promoting the exchange of GDP for GTP. SOS1 recombinant proteins are constructed with the KRAS binding domain and the functional domain (amino acids 594-1049).

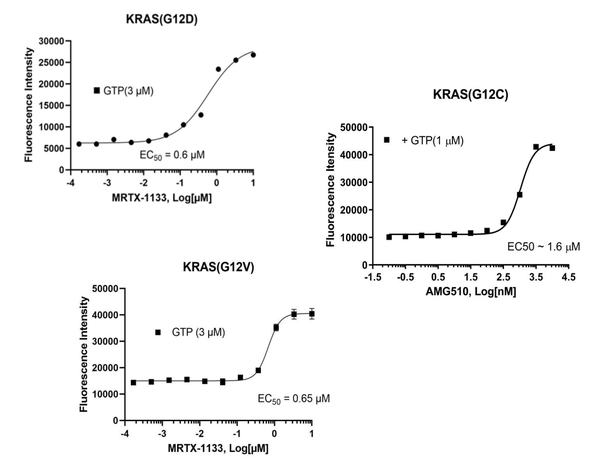
KRAS Nucleotide Exchange Assay Kit

The KRAS Nucleotide Exchange Assay Kits are homogeneous assays that were designed for the screening and profiling of KRAS inhibitors using fluorescent BODIPY®-GDP to monitor nucleotide exchange. Addition of EDTA and GTP in excess pushes the reaction toward the active GTP-KRAS form. KRAS inhibitors block the exchange by locking GDP-KRAS in its inactive, GDP-bound conformation.

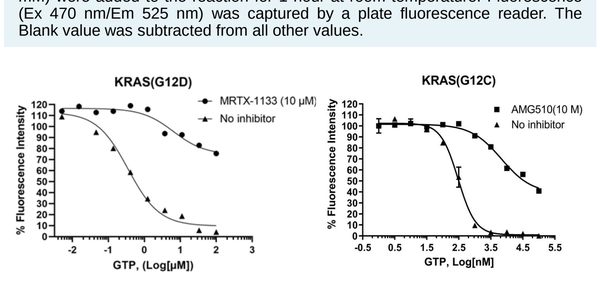
The assays were optimized for two different protocols for greater flexibility of use, either titrating the inhibitor at a fixed GTP concentration or titrating the GTP at a fixed inhibitor concentration.

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.





BODIPY-GDP loaded KRAS was pre-incubated with increasing concentrations of test compound for 2 hours at room temperature. GTP (1 µM) and EDTA (25 mM) were added to the reaction for 1 hour at room temperature. Fluorescence (Ex 470 nm/Em 525 nm) was captured by a plate fluorescence reader. The Blank value was subtracted from all other values.

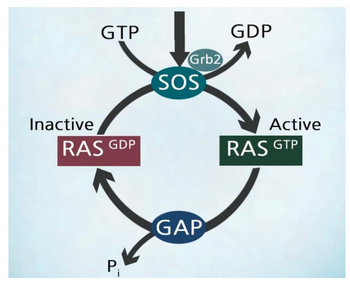


BODIPY-GDP loaded KRAS(G12C) was pre-incubated with test compound AMG510 at a fixed concentration of 10 µM for 2 hours at room temperature. Increasing concentrations of GTP were added. The reaction was initiating immediately by addition of EDTA at 25 mM and incubated for 1 hour at room temperature. Fluorescence (Ex 470 nm/Em 525 nm) was captured by a plate fluorescence. The Blank value was subtracted from all other values.

KRAS proteins were affinity purified, loaded with BODIPY-GDP, and the excess BODIPY-GDP was washed. Protein activity was measured using the KRAS nucleotide exchange assay protocol, with GTP titrated from 0-100 µM (left panels). Protein purity was assessed by 4-20% SDS-PAGE electrophoresis followed by Coomassie Staining (right panels).

KRAS(G12C) Coupled Nucleotide Exchange Assay Kit

GDP-loaded KRAS is inactive and does not interact with downstream effector RAF1 (Serine/Threonine Kinase c-Raf). The KRAS(G12C) Coupled Nucleotide Exchange Assay takes advantage of SOS-mediated nucleotide exchange to activate KRAS(G12C) bound to GDP. Protein SOS1 (Son-of-Sevenless), a guanine nucleotide exchange factor that is recruited by activated growth factor receptors, facilitates nucleotide exchange by forcing the release of GDP from KRAS so that GTP can occupy the nucleotide binding pocket. This results in a conformational change in KRAS that permits its binding to the RBD domain (Ras-Binding Domain) of RAF1, leading to the initiation of a growth-promoting signaling cascade.



Principle: homogeneous AlphaLISA® assay

The KRAS(G12C) Coupled Nucleotide Exchange Assay (BPS Bioscience #78004) uses purified GST-tagged RBD-RAF and His-tagged KRAS(G12C) to monitor the binding of KRAS(G12C) to RBD-RAF in AlphaLISA® format. Glutathione acceptor beads and Nickel-chelate donor beads are brought into proximal range by binding to GST-tagged RBD-RAF and His-tagged KRAS(G12C), respectively, enabling the energy transfer from the donor to acceptor beads after laser excitation. Thus, the donor bead transfers fluorescence excitation to the acceptor bead when KRAS(G12C) forms a complex with RAF1. If no complex is formed, the transfer does not occur.

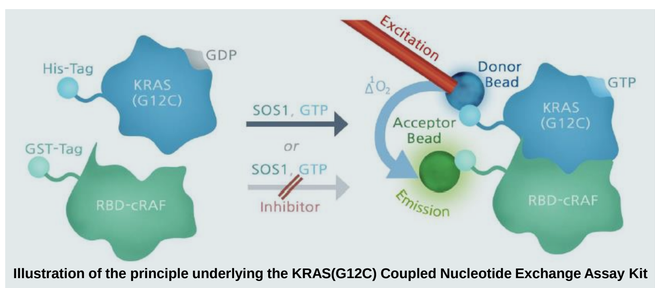
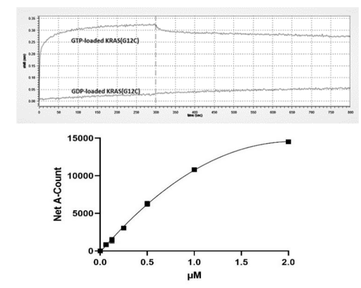
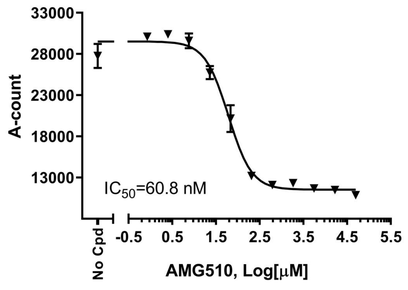


Illustration of the principle underlying the KRAS(G12C) Coupled Nucleotide Exchange Assay Kit



Methods

- GDP-KRAS(G12C), inactive, was pre-incubated with a test inhibitor in the assay buffer.
- Purified SOS1 and an excess of GTP were added to the reaction and incubated for 30 minutes.
- The purified RBD domain of RAF1 was added for 30 minutes.
- Glutathione Acceptor beads (PerkinElmer #AL109C) and Nickel chelate Donor beads (PerkinElmer #AS101D) were added for 30 minutes.
- Data was captured using a compatible Alpha® plate reader (PerkinElmer)

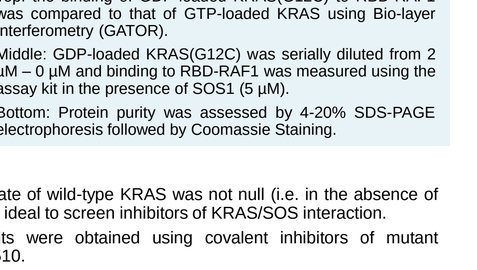


Nucleotide exchange of KRAS(G12C) was evaluated in the presence of increasing concentrations of inhibitor AMG510. Results are expressed as Alpha-count. The negative control consisted of a no-compound condition.

GDP-Loaded KRAS (G12C), His-Tag (#100640)

RBD-RAF, GST-Tag (#100519) (#100519)

SOS1, FLAG-Avi-Tag, Biotin (#100753)



Top: the binding of GDP-loaded KRAS(G12C) to RBD-RAF1 was compared to that of GTP-loaded KRAS using Bio-layer Interferometry (GATOR).

Middle: GDP-loaded KRAS(G12C) was serially diluted from 2 µM – 0 µM and binding to RBD-RAF1 was measured using the assay kit in the presence of SOS1 (5 µM).

Bottom: Protein purity was assessed by 4-20% SDS-PAGE electrophoresis followed by Coomassie Staining.

Results

- The background exchange rate of wild-type KRAS was not null (i.e. in the absence of SOS), therefore this kit is not ideal to screen inhibitors of KRAS/SOS interaction.
- In contrast, excellent results were obtained using covalent inhibitors of mutant **KRAS(G12C)** such as AMG510.

Related products

- Kinase assay kits: EGF receptor (wild-type or mutant), VEGF receptors, FGF receptors (wild-type or mutant).
- SHP-2 homogeneous assay kits (#79330 and #79317).

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Conclusion

Screening and testing new compounds for drug discovery and development requires the use of appropriate tools and assays, which require considerable time and resources to design. BPS Bioscience, scientist-founded and scientist-driven, supports researchers at all phases their research project to accelerate the clinical translation of new treatments for human diseases.

Homogeneous assays tolerate very small volumes, do not necessitate wash steps, and are simple of use, making them ideal for high-throughput screening applications. The two assay kits are useful for the screening and profiling inhibitors of KRAS (wild-type or mutant).

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