RAS nucleotide exchange assays for inhibitor screening and profiling

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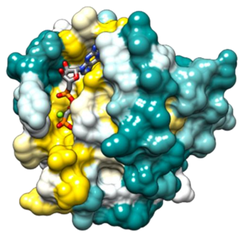
BACKGROUND



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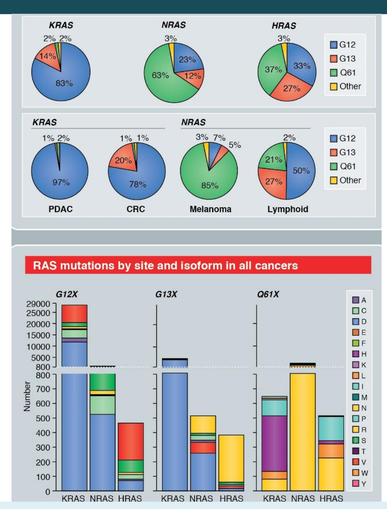
RAS (Rat Sarcoma Virus) is a family of small GTPases involved in cell signal transduction. Like most small GTPases, KRAS binds to GDP in its inactive form and to GTP in its active form. Nucleotide exchange is facilitated by Guanylate exchange factors (GEFs) such as SOS (Son of Sevenless) and gp120 GAP (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.



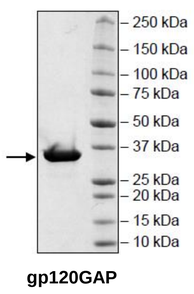
The advancement of these two KRAS(G12C) inhibitors has spurred new efforts in the field. Considering the frequency of RAS mutations in human cancers and the paucity of options for many patients with RAS-induced cancer, there is a large market for pharmaceutical companies to meet a pressing need.

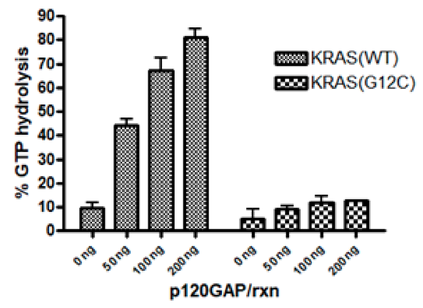
Drug discovery and development projects require reliable assays to screen and evaluate new small molecules that potentially inhibit RAS isoforms. Here we describe the development of two types of assays for high-throughput screening applications and titration of candidate compounds. One assay is based on Alpha®Screen technology, while the other takes advantage of BODIPY™-GDP to allow for either fluorescence polarization or fluorescence intensity data.

Affinity purified proteins

KRAS	Tag	Nucleotide load	Catalog number
KRAS Wild-type	His		11308
KRAS Wild-type	His	BODIPY-GDP	100886
KRAS-G12C	His		100824, 100413
KRAS-G12C	His	GppNHp	100641
KRAS-G12C	His	GDP	100640
KRAS-G12C	His	BODIPY-GDP	100537
KRAS-G12R	His		100825, 100841
KRAS-G12D	His		100623
KRAS-G12D	His	BODIPY-GDP	100887
KRAS-G12V	His		100480
KRAS-G13D	His		100479

The quality of the KRAS nucleotide exchange assay kits depends heavily on the quality of the proteins used in the assay. Wild-type and mutant KRAS were constructed with a His-Tag (6xHis) to allow for affinity purification. Purified proteins can then be loaded with GDP, GppNHp (a non-hydrolyzable GTP analog) or BODIPY-GDP, depending on the application.
Of note, NRAS(Q61H), NRAS(Q61L) and NRAS(Q61K) are also available.





Left panel: gp120GAP purity was assessed by 4-20% SDS-PAGE electrophoresis followed by Coomassie Staining (right panels). Right panel: GTPase activity of KRAS(WT, 50ng) and KRAS(G12C, 100ng) was tested in the presence of increasing concentration of p120GAP (0 ng - 200 ng) and 2 µM GTP by 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 (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

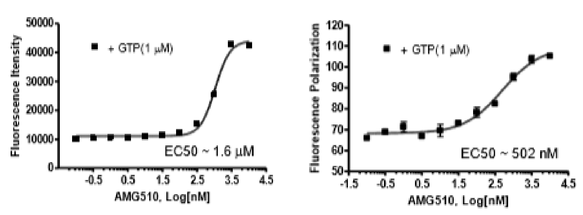
The KRAS Nucleotide Exchange Assay Kit (#79859) is designed for the **screening and profiling of KRAS inhibitors** using fluorescent BODIPY-GDP to monitor nucleotide exchange. Addition of EDTA and of GTP in excess pushes the reaction toward the active GTP-KRAS form. KRAS inhibitors block the exchange by locking GDP-KRAS in its inactive, GDP-bound conformation.

Principle: homogeneous fluorescence polarization assay

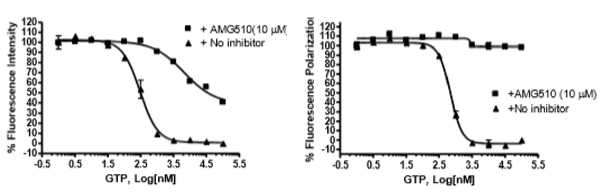
The KRAS Nucleotide Exchange Assay Kit uses protein KRAS (wild-type or mutant), which is affinity purified and loaded with BODIPY-GDP (ThermoFisher), in which the BODIPY® FL fluorophore has been attached to the 2' or 3' position of the ribose ring. BODIPY® FL is a green-fluorescent dye characterized by a high extinction coefficient, high quantum yield and an excited-state lifetime ≥5 nanoseconds. When attached to GDP, the fluorescence of BODIPY is quenched by photoinduced electron transfer from proximal guanosine bases.

In accordance to FP principles, BODIPY-GDP bound to KRAS displays high fluorescence polarization (FP), whereas free BODIPY-GDP displays low FP. Since attachment to GDP quenches the fluorescence of the dye, free GDP-BODIPY emits little fluorescence. In this particular kit, FP and fluorescence intensity can be measured interchangeably.

The kit can be used with two different protocols for greater flexibility:
- GTP titration at a fixed concentration of inhibitor
- Inhibitor titration at a fixed GTP concentration



BODIPY-GDP loaded KRAS(G12C) was pre-incubated with increasing concentrations of test compound AMG510 for 2 hours at room temperature. GTP (1µM) and EDTA (25mM) were added to the reaction for 1 hour at room temperature. Fluorescence (Ex 470nm/Em 525nm) was captured by a plate fluorescence reader (left panel) and FP data was captured using a Tecan FP reader at the same wavelengths (right panel). The Blank value was subtracted from all other values.

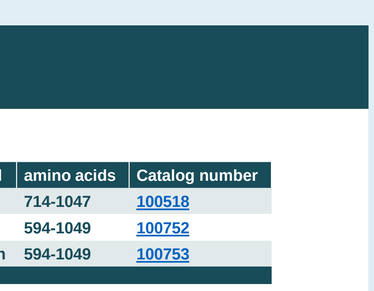


BODIPY-GDP loaded KRAS(G12C) was pre-incubated with test compound AMG510 at a fixed concentration of 10 µM for 2 hours at room temperature. Increasing concentrations of GTP were added. The reaction was initiating immediately by addition of EDTA at 25mM and incubated for 1 hour at room temperature. Fluorescence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

KRAS proteins were affinity purified, loaded with BODIPY-GDP, and the BODIPY-GDP was removed. Protein activity was measured using the KRAS nucleotide exchange assay kit (#79857), with GTP titrated in 3-fold serial dilution 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 kit takes advantage of SOS-mediated nucleotide exchange as a way to activate KRAS(G12C) bound to GDP. Protein SOS1, a guanine nucleotide exchange factor that is recruited by growth factor receptors upon their activation, 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 Alpha® assay

The KRAS(G12C) Coupled Nucleotide Exchange Assay Kit (#78004) uses GST-tagged RBD-RAF and His-tagged KRAS(G12C) to monitor the binding of KRAS(G12C) to RBD-RAF in the Alpha® assay. 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. If KRAS(G12C) forms a complex with RAF1, the donor bead transfers fluorescence excitation to the acceptor beads. If no complex is formed, the transfer does not occur.

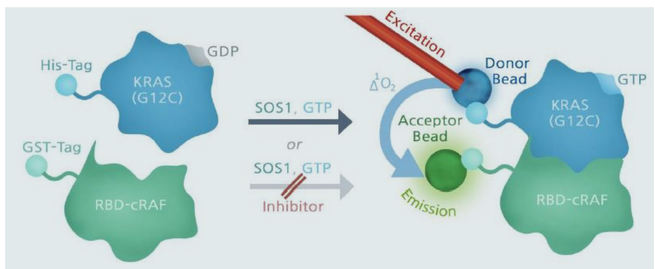
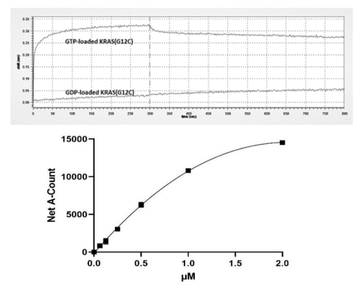
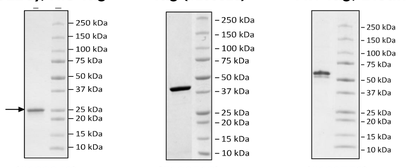


Illustration of the principle underlying the KRAS(G12C) Coupled Nucleotide Exchange Assay Kit



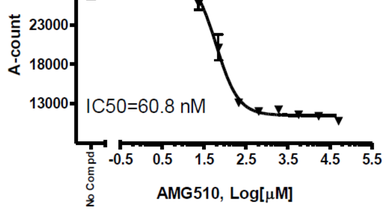
GDP-Loaded KRAS (G12C), His-Tag (#100640) | RBD-RAF, GST-Tag (#100519) | SOS1, FLAG-Avi-Tag, Biotin (#100753)



Proteins were purified according to their tag. Top: the binding of GDP-loaded KRAS(G12C) binding to RBD-RAF1 was compared to that of GTP-loaded KRAS for 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 Alpha® assay kit in the presence of SOS1 (5 µM). Bottom: Protein purity was assessed by 4-20% SDS-PAGE electrophoresis followed by Coomassie Staining.

Methods

- GDP-KRAS(G12C), inactive, is pre-incubated with a test inhibitor in the assay buffer
- Purified SOS1 and an excess of GTP are added to the reaction and incubated for 30min
- The purified RBD portion of RAF1 is added and incubated for 30min
- Glutathione Acceptor beads (PerkinElmer #AL109C) and Nickel chelate Donor beads (PerkinElmer #AS101D) are added and incubated for 30min
- Data is 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 in term of Alpha-count. The negative control consisted of a no-compound condition.

Results

- It was observed that the background exchange rate of wild-type KRAS is 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)

Conclusion

Screening and testing new compounds in drug discovery and development phases 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.

FP and Alpha® assays tolerate very small volumes. Both are in-solution homogenous assays that 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 of KRAS (wild-type or mutant)) inhibitors.