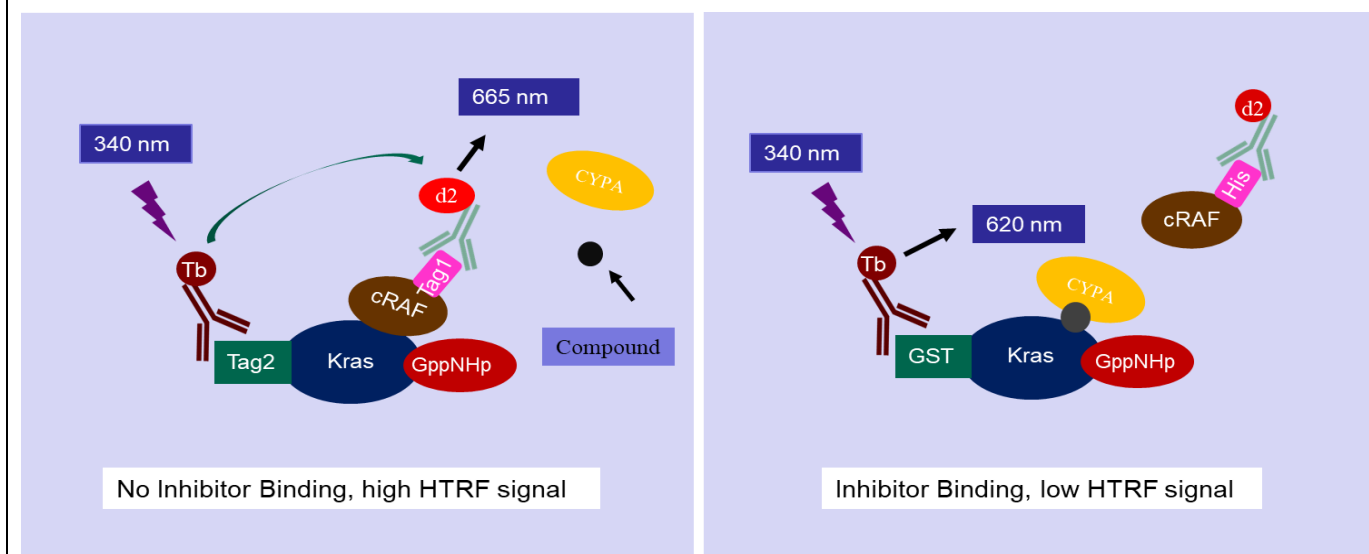


Background

Kras is a member of the RAS protein family, which are a class of small GTPases involved in cell signaling pathways. The Ras signaling pathway regulates diverse cellular processes, including cell proliferation, differentiation, and survival. Conversion of Ras from the inactive GDP-bound state to the active GTP-bound state activates the downstream effector and promotes cell growth. RAF is a key downstream effector of RAS. Since the frequently mutated *Ras* genes are associated with various human tumors, the Ras-RAF signaling pathway is considered an important therapeutic target for cancer treatment. However, Ras is considered undruggable since it lacks suitable binding pockets on the surface. Recently, a discovery of a small molecule inhibitor blocks Ras-RAF signaling pathway by remodeling Cyclophilin A (CYPA) and forming a CYPA:drug:KRAS ternary complex. This inhibitory strategy provides a new method for developing drugs targeting Kras for treatment of cancers.

Assay Principle

The Kras (G12D) Inhibitor assay kit is a TR-FRET based assay, which is designed to screen Kras inhibitors and determine the Kras-inhibitor binding affinity. Tag2-Kras (G12D) in this assay kit is loaded with GppNHp, which represents the activated Kras. The Ras binding domain (RBD) of cRAF has a Tag1 at N-terminus. A Terbium-labeled anti-Tag2 antibody binding to the Tag2-Kras serves as a fluorescence donor (HTRF donor), activation of which results in fluorescence resonance energy transfer (FRET) if Tag1-cRAF binds to the Kras, since the binding brings Terbium on the anti-Tag2 antibody close to the fluorophore on the anti-Tag1 antibody (HTRF acceptor). Thus, the binding status can be quantitatively measured by calculating the ratio of the emission fluorescence intensity of the acceptor (665 nm) and donor (620 nm). If an inhibitor associated with CYPA binds to the Kras and blocks the cRAF binding, the HTRF signal will be reduced.



Application

High throughput screening of compounds that inhibit the binding between activated Kras (G12D) and cRAF for drug discovery.

Plate Reader

A HTRF® certified microplate reader capable of measuring Time Resolved Fluorescence Resonance Energy Transfer (TR-FRET) is required.

Components

| Catalog number | Item | Amount | Storage |
|----------------|---|--------|------------------|
| 5727-CK-B | 2X Kras Binding buffer | 25 mL | -20°C |
| 5727-4123-T2P | Recombinant human Tag2-Kras (G12D), GppNHp loaded | 22µL | -80°C |
| 5727-2972 | Recombinant Cyclophilin A (CYPA) | 200 µL | -80°C |
| 7237231-T1 | Recombinant human Tag1-cRAF, RBD | 5 µL | -80°C |
| 37882 | Terbium-labeled anti-Tag2 antibody | 20 µL | -80°C |
| 44732 | Fluorescence labeled anti-Tag1 antibody | 20 µL | -80°C |
| | 384-well microplate | 1 | Room temperature |

Materials needed but not supplied

1. Microplate reader, HTRF® certified microplate reader (such as Tecan M1000, Tecan Spark, etc.)
2. 0.5 M DTT
3. Adjustable micro-pipettor
4. Sterile Tips

Assay protocol

1. Prepare compound dilution buffer containing 2 mM DTT (CD buffer)

For example, mix 250 μ l of 2X Kras Binding Buffer, 178 μ l of distilled water, 70 μ l of CYPA and 2 μ l of 0.5 M DTT. Make only enough CD buffer as needed for the assay. Store the remaining Binding buffer at -20°C.

2. Prepare the inhibitor compound solution

If the inhibitor compound is dissolved in water, make a solution of the compound 10-fold higher than the final concentration in CD buffer (since you will add 2 μ l to the 20 μ l reaction).

If the inhibitor compound is dissolved in DMSO, make a 100-fold higher concentration of the compound than the highest concentration you want to test in DMSO. Then make a 10-fold dilution in CD buffer (at this step, the compound concentration is 10-fold higher than the final concentration and the DMSO concentration is 10%). To determine an IC₅₀ or to test lower concentrations of the compound, prepare a series of further dilutions in CD buffer containing 10% DMSO (the final concentration of the DMSO will be 1% in all samples).

3. Prepare 1X Assay Buffer containing 2 mM DTT (AB buffer)

For example, mix 500 μ l of 2X Kras Binding Buffer, 496 μ l of distilled water and 4 μ l of 0.5 M DTT. Make only enough AB buffer as needed for the assay. Store the remaining Binding buffer at -20°C.

4. Prepare Kras (G12D) solution

Thaw Kras protein on ice. Upon first thaw, briefly spin tube to recover the full contents at the bottom of the tube. Make aliquots of the enzyme for single use. Store remaining undiluted enzyme at -80°C.

Note: Kras protein is sensitive to freeze/thaw cycles. Limit number freeze-thaw cycles for best results. Do not re-use the diluted protein.

Dilute the Kras protein 75-fold (1 μ L Kras G12D + 74 μ L AB buffer).

Add 4 μ l of diluted protein solution to each positive control well and inhibitor test well.

Add 4 μ l of AB buffer to each of negative control well.

5. Add inhibitor

Add 2 μ l of diluted compound solution to each inhibitor test well.

Add 2 μ l of CD buffer to each of negative and positive control well.

6. Prepare cRAF solution

Thaw cRAF protein on ice. Upon first thaw, briefly spin tube to recover the full contents at the bottom of the tube. Make aliquots of the enzyme for single use. Store remaining undiluted protein at -80°C.

Note: cRAF protein is sensitive to freeze/thaw cycles. Limit number freeze-thaw cycles for best results. Do not re-use the diluted protein.

Dilute the cRAF protein 400-fold (1 µL cRAF + 399 µL of **AB buffer**).

Add 4 µl of diluted protein solution to each well.

7. Prepare dye solution

Dilute Terbium-labeled anti-Tag2 antibody and fluorescence-labeled anti-Tag1 antibody 1:200 in **AB buffer**. For example: 1 µl of Terbium-labeled anti-Tag2 antibody + 1 µl of fluorescence-labeled anti-Tag1 antibody + 198 µl of **AB buffer**.

Add 10 µl of this dye mixture to each well.

8. Incubate the reaction at room temperature for 30 minutes.

9. Measure fluorescent intensity

HTRF compatible microplate reader is needed to measure fluorescent intensity of the samples. Fluorescent intensity should be measured twice:

1. Excitation wavelength at 340 nm and emission at 620 nm.
2. Excitation wavelength at 340 nm and emission at 665 nm.

Protocol Summary

| Component | Negative Control | Positive Control | Inhibitor Test |
|---------------------|------------------|------------------|----------------|
| AB buffer | 4 µl | | |
| Kras (G12D) protein | | 4 µl | 4 µl |
| CD Buffer | 2 µl | 2 µl | |
| Inhibitor solution | | | 2 µl |
| cRAF protein | 4 µl | 4 µl | 4 µl |
| Dye solution | 10 µl | 10 µl | 10 µl |
| Total Volume | 20 µl | 20 µl | 20 µl |

Incubate at room temperature for 30 minutes.

Data Analysis

1. Calculate sample HTRF signal of each well.

$$HTRF = \frac{\text{Fluorescent intensity at 665 nm}}{\text{Fluorescent intensity at 620 nm}} \times 10,000$$

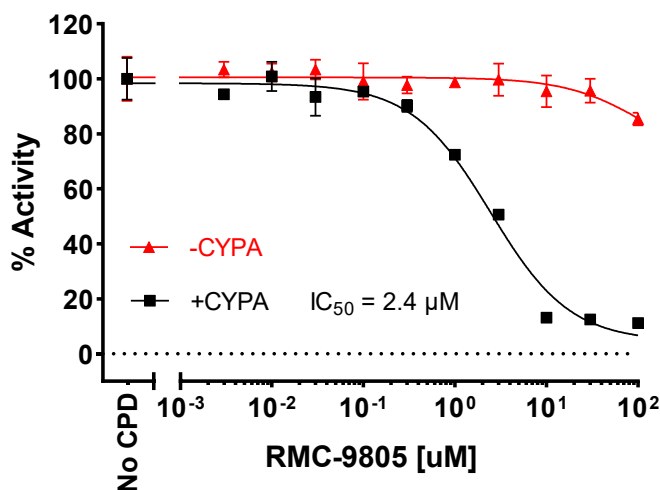
2. Calculate percentage activity

In the absence of the compound (positive control), the sample signal (P) is defined as 100% activity. In the absence of enzyme (negative control), the sample signal (N) is defined as 0% activity. The percent activity in the presence of each compound is calculated according to the following equation: % activity = (S-N)/(P-N) X100, where S= the sample signal in the presence of the compound.

$$\% \text{ Activity} = \frac{S - N}{P - N} \times 100$$

Assay result

Kras (G12D)-cRAF Binding



Related products:

| <u>Product Name</u> | <u>Catalog #</u> | <u>Size</u> |
|-----------------------------------|------------------|---------------|
| Kras Wild Type (WT), GST-tag | 5727-4121G | 50 µg, 100 µg |
| Kras WT, GST-tag, GDP Loaded | 5727-WTG-G | 50 µg, 100 µg |
| Kras WT, GST-tag, GppNHp loaded | 5727-WTG-GP | 50 µg, 100 µg |
| Kras G12C, His -tag | 5727-4122H | 50 µg, 100 µg |
| Kras G12C, GST-tag | 5727-4122G | 50 µg, 100 µg |
| Kras G12C, GST-tag, GDP Loaded | 5727-4122G -G | 50 µg, 100 µg |
| Kras G12C, GST-tag, GppNHp loaded | 5727-4122G -GP | 50 µg, 100 µg |
| Kras G12D, GST-tag | 5727-4123G | 50 µg, 100 µg |

| | | |
|--|----------------|-----------------------------|
| Kras G12D, GST-tag, GDP Loaded | 5727-4123G -G | 50 µg, 100 µg |
| Kras G12D, GST-tag, GppNHP loaded | 5727-4123G -GP | 50 µg, 100 µg |
| Kras G12R, GST-tag, | 5727-4127G | 50 µg, 100 µg |
| Kras G12R, GST-tag, GDP Loaded | 5727-4127G -G | 50 µg, 100 µg |
| Kras G12R, GST-tag, GppNHP loaded | 5727-4127G -GP | 50 µg, 100 µg |
| Kras G12V, GST-tag, | 5727-4128G | 50 µg, 100 µg |
| Kras G12V, GST-tag, GDP Loaded | 5727-4128G -G | 50 µg, 100 µg |
| Kras G12V, GST-tag, GppNHP loaded | 5727-4128G -GP | 50 µg, 100 µg |
| Kras G13D, GST-tag | 5727-4133G | 50 µg, 100 µg |
| Kras G13D, GST-tag, GDP Loaded | 5727-4133G -G | 50 µg, 100 µg |
| Kras G13D, GST-tag, GppNHP loaded | 5727-4133G -GP | 50 µg, 100 µg |
| Human RBD-RAF1, N-His tag, C-FLAG tag | 7237231 | 50 µg, 100 µg |
| Human SOS1, No tag | 7671 | 50 µg, 100 µg |
| Human SOS1, Avi-His tag | 7671HA | 50 µg, 100 µg |
| | | |
| Kras WT Nucleotide Exchange Assay Kit | 5727-4121NK | 384 reactions |
| Kras G12C Nucleotide Exchange Assay Kit | 5727-4122NK | 384 reactions |
| Kras G12D Nucleotide Exchange Assay Kit | 5727-4123NK | 384 reactions |
| Kras G12R Nucleotide Exchange Assay Kit | 5727-4127NK | 384 reactions |
| Kras G12V Nucleotide Exchange Assay Kit | 5727-4128NK | 384 reactions |
| Kras G13D Nucleotide Exchange Assay Kit | 5727-4133NK | 384 reactions |
| | | |
| Kras WT – cRAF Binding Assay Kit | 5727-4121BK | 384 reactions |
| Kras G12C – cRAF Binding Assay Kit | 5727-4122BK | 384 reactions |
| Kras G12C– cRAF Binding Assay Kit | 5727-4123BK | 384 reactions |
| Kras G12R– cRAF Binding Assay Kit | 5727-4127BK | 384 reactions |
| Kras G12V – cRAF Binding Assay Kit | 5727-4128BK | 384 reactions |
| | | |
| Kras WT/cRAF/CYPA/Inhibitor Assay Kit | 5727-4121CK | 384 reactions |
| Kras G12C/cRAF/CYPA/Inhibitor Assay Kit | 5727-4122CK | 384 reactions |
| Kras G12D/cRAF/CYPA/Inhibitor Assay Kit | 5727-4123CK | 384 reactions |
| Kras G12V/cRAF/CYPA/Inhibitor Assay Kit | 5727-4128CK | 384 reactions |
| Kras G13D/cRAF/CYPA/Inhibitor Assay Kit | 5727-4133CK | 384 reactions |
| | | |
| TEV Protease | 190001 | 1,000 Units, 10,000 Units |
| TEV Protease- His-tag | 190001-R | 50 ug, 200 ug, 1 mg |
| PreScission Protease (HRV 3C) | 190002 | 1,000 units, 10,000 units |
| Recombinant SUMO Protease (Ulp1) | 190003 | 1,000 units, 10,000 units |
| Recombinant YopH | 200100 | 10 ug, 20 ug, 100 ug, 1 mg |
| Recombinant Biotin Protein Ligase (BirA) | 90101 | 100 ug |
| Recombinant SortaseA-5M | 90201 | 50 ug, 200ug |
| Recombinant Mouse Leukemia Inhibitory Factor | 11-0001 | 10 ug, 100 ug |
| Recombinant Human LIF | 12-0002 | 10 ug, 100 ug, 1 mg |
| Recombinant Human FGF-basic, Carrier-free | 12-0005CFR | 50 ug, 100 ug, 500 ug, 1 mg |

Products are for research use only and are not intended for human use. We do not sell to patients.