Kras (WT)/cRAF/CYPA/Inhibitor Assay Kit

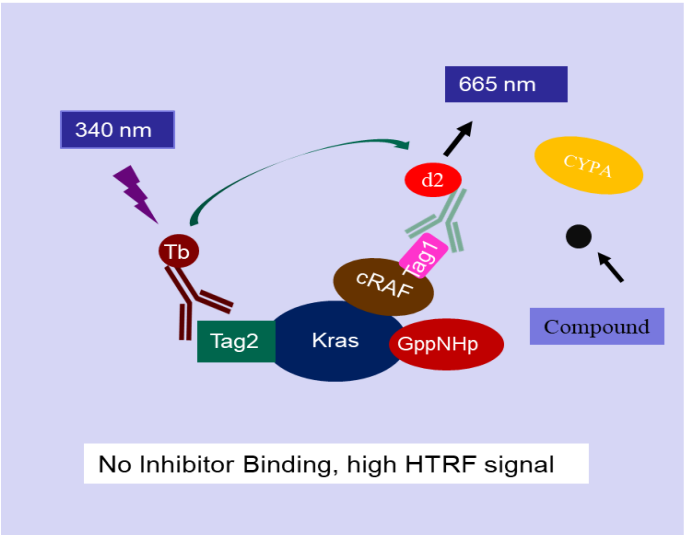
Catalog Number: 5272-4121CK

Background

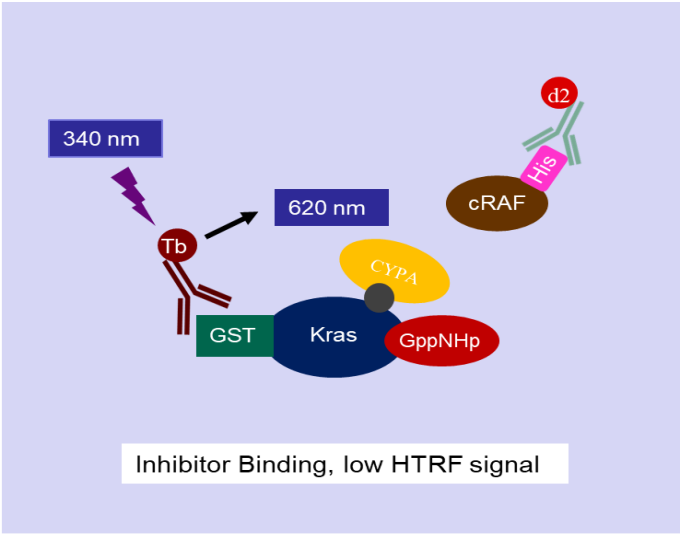
Kras is a member of the RAS protein family, which are a class of small GTPases involved in cell signaling pathways. The Ras signaling pathway regulates diverse cellular processes, including cell proliferation, differentiation, and survival. Conversion of Ras from the inactive GDP-bound state to the active 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 remolding 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 (WT) 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 (WT) in this assay kit is loaded with GppNHp, which represents the activated Kras. The Ras binding domain (RBD) of cRAF in the kit 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 quantitively 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.



No Inhibitor Binding, high HTRF signal



Inhibitor Binding, low HTRF signal

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Application

High throughput screening of compounds that inhibit the binding between activated Kras (WT) 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
5727-4121-T2P	Recombinant human Tag2-Kras (WT), GppNHp loaded	15 µL	-80°C
5272-2972	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 µl of 2X Kras Binding Buffer, 178 µl of distilled water, 70 µl of CYPA and 2 µl 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 µl to the 20 µl 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 as 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 µl of 2X Kras Binding Buffer, 496 µl of distilled water and 4 µl 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 (WT) 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 110-fold (1µL Kras WT + 109 µL AB buffer).

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

   Add 4 µl of AB buffer to each of negative control well.

5. Add inhibitor

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

   Add 2 µl 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 (WT) 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.			

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Data Analysis

1. Calculate sample HTRF signal of each well.

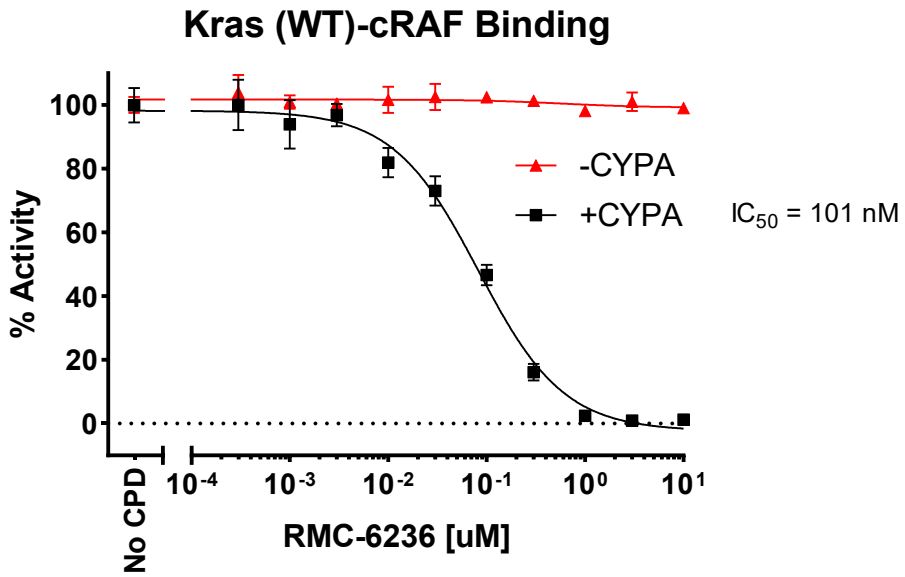
$$HTRF = \frac{\text{Fluorescent intensity at 665 nm}}{\text{Fluorescent intensity at 620 nm}} X10{,}000$$

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.

$$\%\ \text{Activity} = \frac{S - N}{\text{P} - \text{N}} X100$$

Assay result



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