Application of NanoBret Target Engagement Cellular Assay for Measurement of Inhibitor Binding to Wild Type and Mutant RAS in Live Cells

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Abstract

NanoBRET™ target engagement (TE) is the first biophysical technique that broadly enables the quantitative determination of protein inhibitor occupancy in live cells without disruption of cellular membrane integrity. This quantitative capability is achieved in live cells via BRET with an optimized set of cell-permeable tracers, allowing the measurement of compound binding to selected cellular target proteins. RAS is a well-known oncogene that is frequently mutated in most lung, pancreatic, and colorectal cancers and is associated with poor disease prognosis. Mutated RAS is locked in the activated GTP bound state and facilitates enhanced RAS signaling in cancer cells. While a desirable target, the absence of good druggable binding pockets has made modulator compound discovery challenging and unsuccessful. The recent discovery of a unique switch II binding pocket and successful inhibition of the KRAS (G12C) mutant by covalent inhibitors have led to the resurgence of interest in the design of inhibitors targeting RAS directly. Here, we performed NanoBRET™ TE cellular assay with RAS inhibitors against transfected RAS and its mutants. Our data demonstrate that NanoBRET™ TE cellular assay can measure the apparent affinity of RAS inhibitors by competitive displacement of a NanoBRET™ RAS switch I/II pocket tracer, reversibly bound to the LgBiT- and SmBiT-KRAS, KRAS(G12C), KRAS(G12D), KRAS(G12V), KRAS(G13D), KRAS(Q61H), KRAS(Q61L), KRAS(Q61R), or HRAS fusion constructs co-transfected in live HEK293 cells. Z'-factor analysis indicates the assay is High Throughput Screening (HTS) compatible and reproducible. In addition, we are able to confirm the downstream signaling inhibition of phospho-ERK1/2 by KRAS(G12C)-specific inhibitors AMG510 (Sotorasib), ARS-1620, MRTX849, and MRTX1257 in MIA PaCa-2 and SW837 cells bearing KRAS(G12C) mutation by Western blot assay. Our results suggest NanoBRET™ TE cellular assay can serve as a great tool to facilitate RAS pathway drug discovery against human cancers.

Introduction
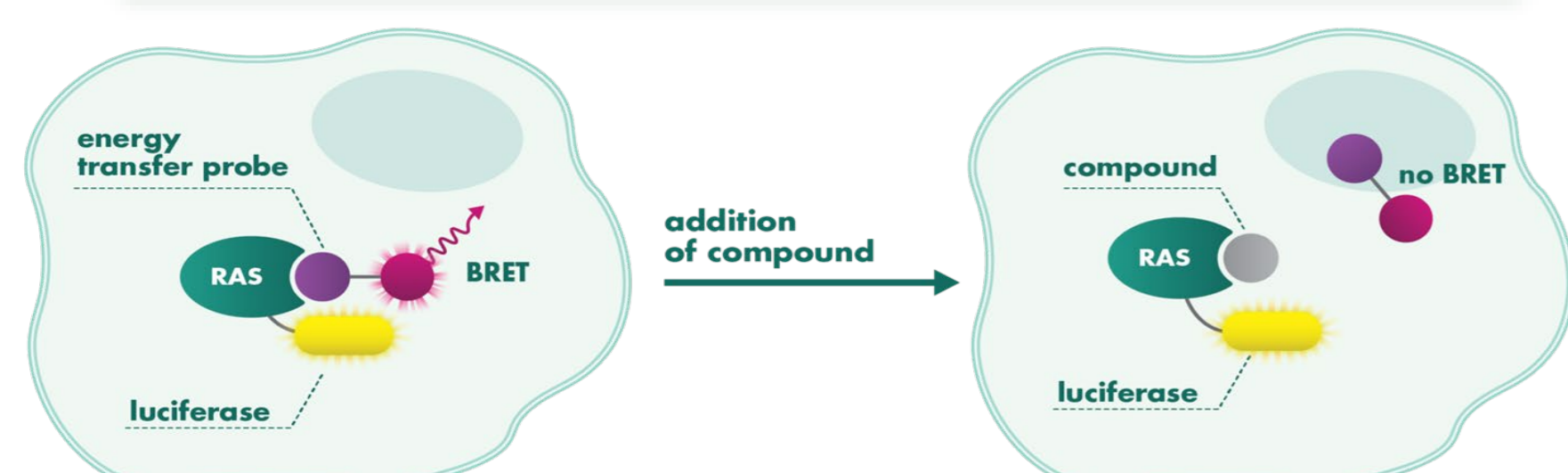
The NanoBRET TE Assay analyzes the apparent binding affinity and permeability of test compounds by competitive displacement of a NanoBRET tracer reversibly bound to a NanoLuc or NanoBiT fusion protein in cells. Binding of the test compound results in the loss of NanoBRET signal between the target protein and the tracer in intact cells.
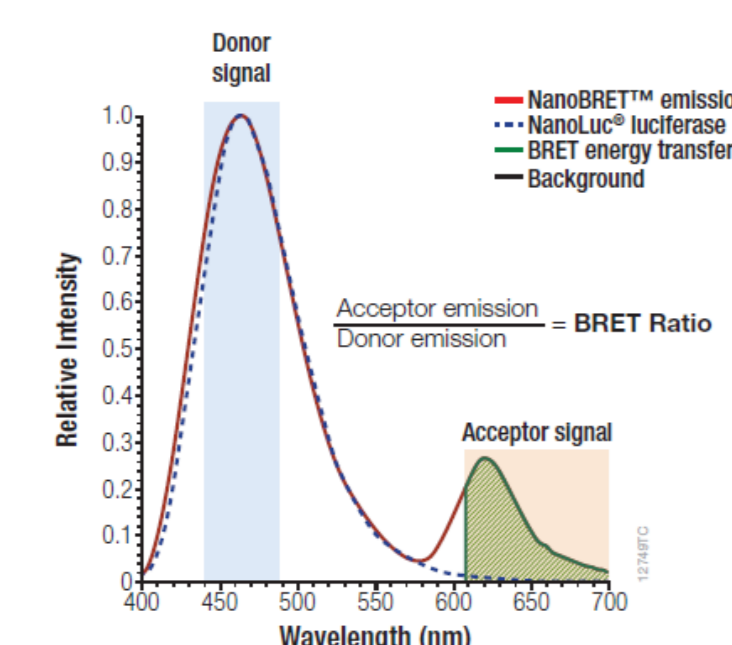
Experimental Procedures

Materials
HEK293, MIA PaCa-2, and SW837 cell lines were purchased from ATCC. FuGENE HD Transfection Reagent, Large BiT and Small BiT RAS fusion plasmids, Transfection Carrier DNA, NanoBRET RAS Tracer K1, and tracer dilution buffer, NanoBRET Nano-Glo Substrate were obtained from Promega. Phospho-ERK1/2 (Thr202/Tyr204) antibody and PathScan® Phospho-ERK1/2 (Thr202/Tyr204) Sandwich ELISA kits were purchased from Cell Signaling Technology. All other reagents were purchased from reliable vendors.

NanoBRET™ Target Engagement Cellular RAS Assay
Assays were conducted following the Promega assay protocol with some modifications. HEK293 cells were transiently transfected with Large BiT and Small BiT RAS Fusion Vector DNA by FuGENE HD Transfection Reagent. Test compounds were delivered into a 384 well assay plate by Echo 550 (Labcyte Inc). Transfected cells were harvested and mixed with NanoBRET Tracer Reagent and dispensed into a 384 well plate and incubated at 37°C in a 5% CO_2 cell culture incubator for 2 hours. The NanoBRET Nano-Glo Substrate was added into the wells of the assay plate and incubated for 2–3 minutes at room temperature. The donor emission wavelength (460nm) and acceptor emission wavelength (600nm) were measured in EnVision plate reader. The BRET ratio was calculated. BRET Ratio = [(Acceptor sample ÷ Donor sample) − (Acceptor no-tracer control ÷ Donor no-tracer control)]. The IC50 values of compounds were calculated with the GraphPad Prism program.

Western Blot
MIA PaCa-2 and SW837 cells were treated with reference compound ARS-1620 for 1.5 hours and then stimulated with 1 μM PMA for 30 mins. The cells were lysed with 1x cell lysis buffer. Cell lysate samples were subjected to SDS-PAGE with 12% Bis-Tris gel and transferred onto nitrocellulose membranes by iBlot dry blotting system. The membranes were blocked with 3% milk, probed with phospho-ERK1/2 (Thr202/Tyr204) antibody, and re-probed with α-Tubulin antibody. Anti-rabbit IgG IRDye 680RD and anti-mouse IgG IRDye 800CW secondary antibodies were used to detect the primary antibodies. The membranes were scanned with LI-COR Odyssey Fc Imaging System.

ELISA
SW837 cells were seeded in the wells of a 96-well tissue culture plate overnight. Cells were treated with ARS-1620 (starting at 10 μM, 10-dose with 3-fold dilution) for 2 hours. The cells were washed with PBS and lysed with 1x cell signaling lysis buffer. Phospho-ERK1/2 (Thr202/Tyr204) in SW837 cells was measured with a standard ELISA assay protocol from Cell Signaling Technology. The IC_{50} curve was plotted and IC50 value was calculated using the GraphPad Prism program based on a sigmoidal dose-response equation.

1. Results

1. NanoBRET™ target engagement cellular RAS assay is suitable for high throughput screening in 384-well plate format
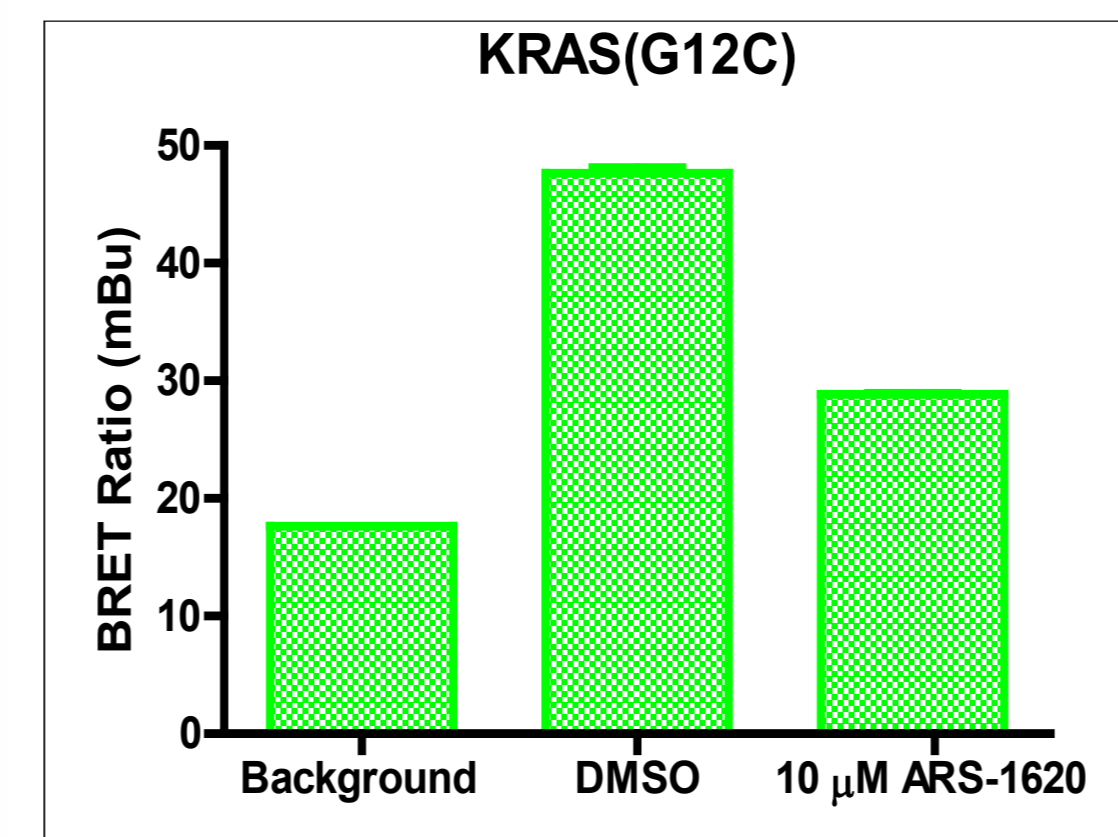
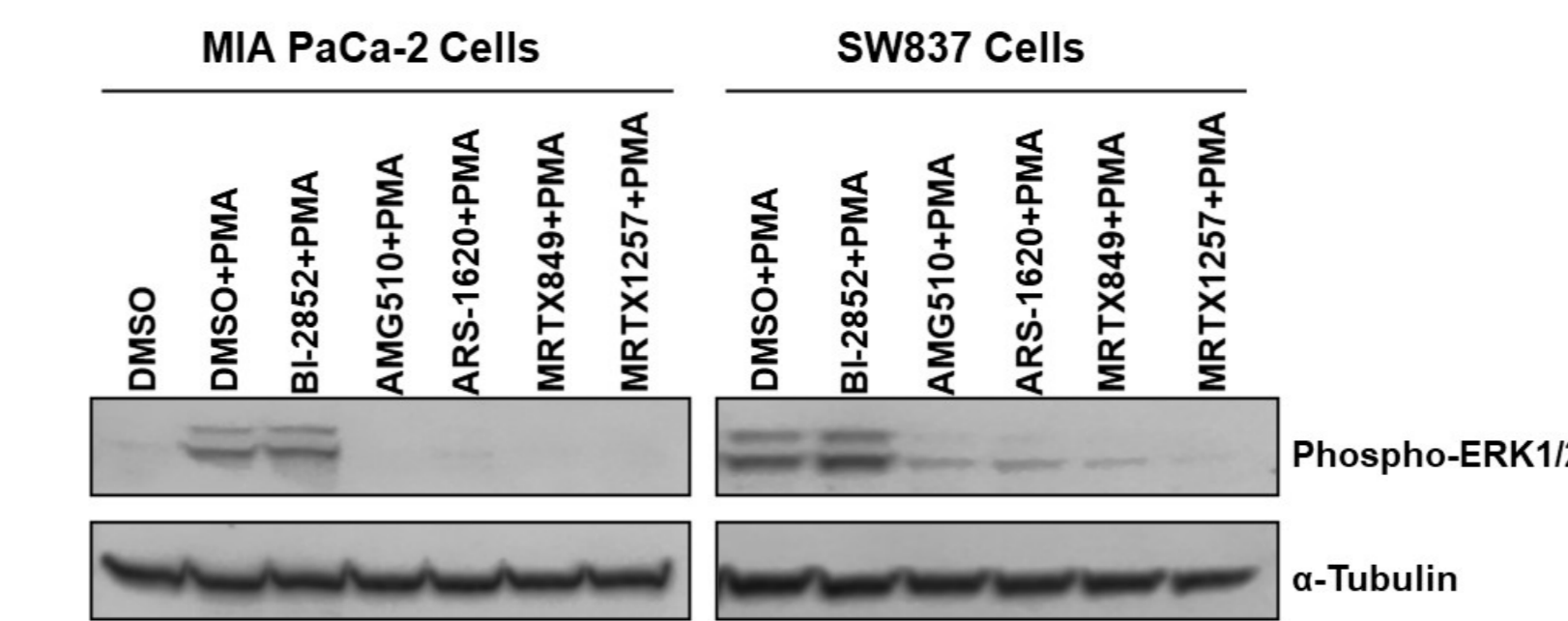Sample# (N)=36
S:B=2.70
Z'-factor=0.62

*4000 cells /well in 384-well assay plate

*Compound treatment time: 2 hours

*Tracer concentration: RAS tracer K1 @ 2 μM

2. Results

2. Representative data of reference compounds in NanoBRET target engagement cellular assay for wild type and mutant RAS

*4000 cells / well in 384-well assay plate   *Compound treatment time: 2 hours   *Tracer concentration: RAS tracer K1 @ 2μM

3. Results

3. KRAS(G12C) inhibitors block ERK1/2 phosphorylation in MIA PaCa-2 and SW837 cells bearing KRAS(G12C) mutation with Western blot assay
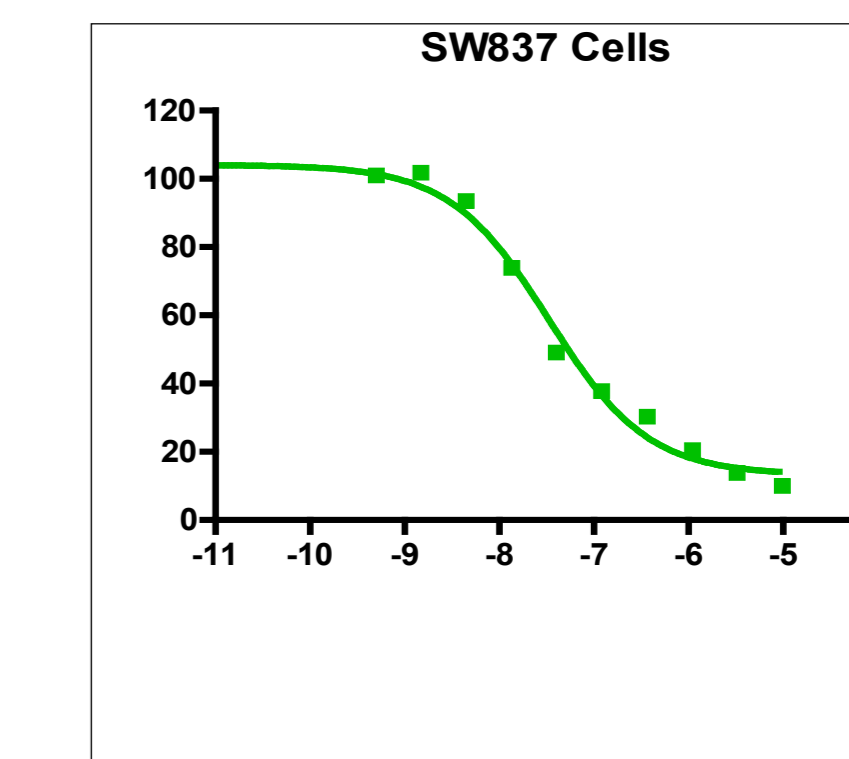

*70000 cells / well in 24-well assay plate

*Treatment Time: Inhibitors for 1.5 hrs, and then PMA for 30 min

*Antibody: Phospho-ERK1/2 (Thr202/Tyr204) antibody

4. Results

4. KRAS(G12C) inhibitor ARS-1620 blocks ERK1/2 phosphorylation in SW837 cells bearing KRAS(G12C) mutation with ELISA
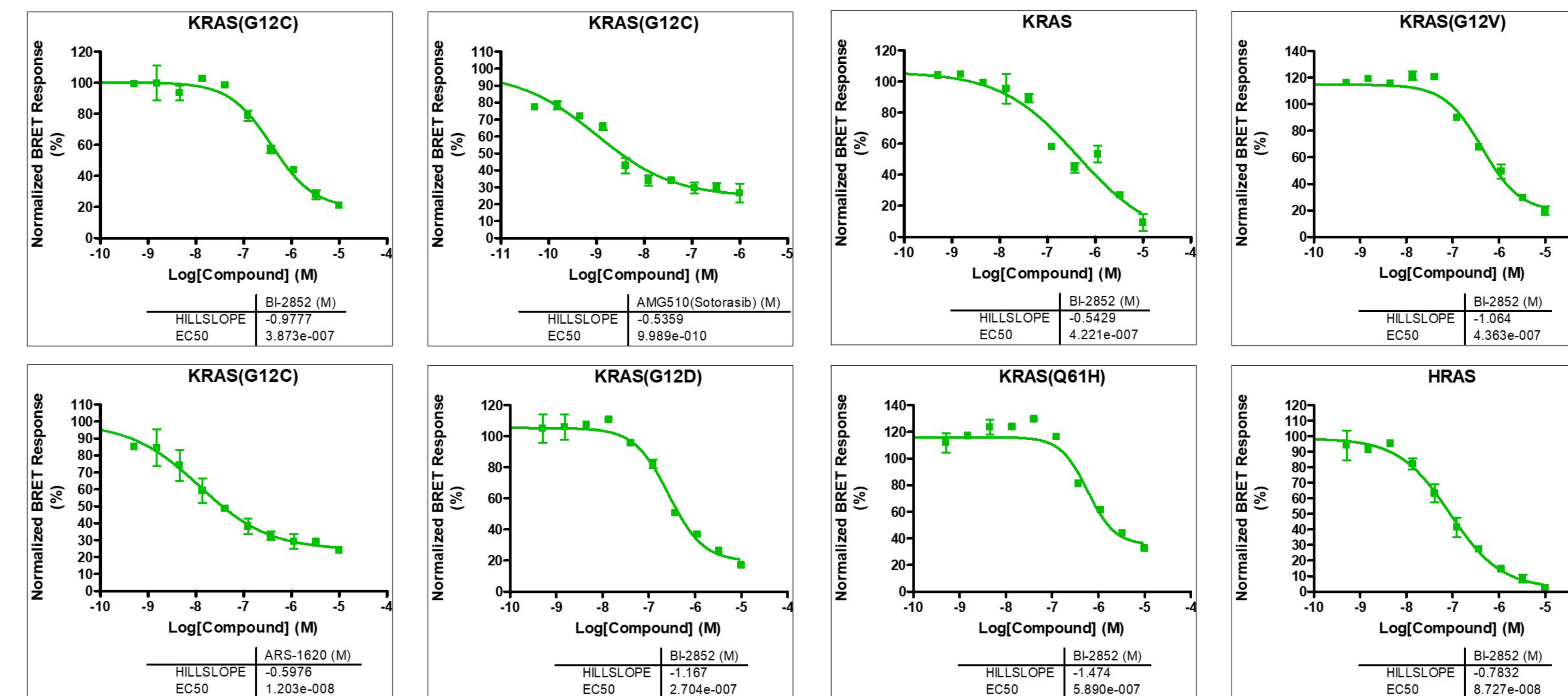

*15000 cells / well in 96-well assay plate

*Treatment time: ARS-1620 for 2 hrs

*ELISA: Pathscan® Phospho-ERK1/2 (Thr202/Tyr204) Sandwich ELISA kit

Summary

NanoBRET target engagement cellular RAS assay has been successfully validated and optimized in a 384-well plate format in live cells.

The assay is suitable for high throughput screening to identify RAS inhibitors in live cells with Z'-factor > 0.6 and Assay S/B ratio >2 .7 folds.

IC50 measurement of reference compounds against wild type and mutant RAS demonstrates the value of using a live cell target engagement assay platform for compound profiling.

The inhibitory effect of clinical RAS pathway drugs on downstream ERK1/2 phosphorylation is confirmed by both Western blot and ELISA assay formats.

Reaction Biology now offers NanoBret target engagement cellular assay service for RAS wild type and RAS mutants for compound screening.

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