Table 1 List of Donors and Acceptors Constructs Available for BRET2 RAS Biosensors

RLuc8 donor constructs	GFP^2 acceptor constructs
$KRAS^{WT}$	iDAb RAS
$KRAS^{G12A}$	$iDAb_{dm}$ RAS
$KRAS^{G12C}$	iDAb control (iDAb LMO2)
$KRAS^{G12D}$	PI3Kα RBD
$KRAS^{G12R}$	PI3Kγ RBD
$KRAS^{G12V}$	CRAF RBD
$KRAS^{S17N}$	RALGDS RA
$NRAS^{WT}$	$CRAF^{S257L\ FL}$
$NRAS^{Q61H}$	$PI3K\alpha^{FL}$ ($\pm p85\alpha^{FL}$)
$HRAS^{WT}$	
$HRAS^{G12V}$	
LMO2	$iDAb_{dm}$ LMO2

Table 2 DNA Mix for a Typical BRET2 Titration Curve Experiment[a]

RLuc8 constructs (ng)	GFP^2 constructs (ng)	pEF-myc-cyto empty (ng)	RLuc8:GFP^2 constructs ratio
50	0	1550	1:0
	12.5	1537.5	1:0.25
	25	1525	1:0.5
	50	1500	1:1
	100	1450	1:2
	250	1300	1:5
	500	1050	1:10
	750	800	1:15
	1000	550	1:20

[a]Quantities are indicated for transfection of one well of a 6-well plate.

Table 3 List of Competitor Constructs Available for BRET2 Competition Assay

Positive competitors	Negative competitors
Intracellular Domain Antibodies (iDAb) competitors	
pEF-membrane-FLAG-iDAb RAS-myc	pEF-membrane-FLAG-iDAb LMO2-myc
pEF-iDAb RAS-myc	pEF-iDAb LMO2-myc
Designed Ankyrin Repeat Proteins (DARPins) competitors	
pEF-DARPin K27-myc	pEF-DARPin E3.5-myc
pEF-DARPin K55-myc	

Table 4 Ratio of DNA Used for the Competition Assays With iDAbs or DARPins

RLuc8 constructs (DNA ng)	GFP^2 constructs (DNA ng)	RLuc8:GFP^2 constructs ratio	Competitor constructs (DNA ng)	pEF-myc-cyto empty (ng)
RAS (50 ng)			100	1300
	PI3Kα RBD (150 ng)	1:3	500	900
			1000	400
			100	1350
	PI3Kγ RBD (100 ng)	1:2	500	950
			1000	450
			100	1350
	CRAF RBD (100 ng)	1:2	500	950
			1000	450
			100	1350
	RALGDS RA (100 ng)	1:2	500	950
			1000	450
			100	1400
	$CRAF^{FL}$ (50 ng)	1:1	500	1000
			1000	500
			100	1200
	$PI3K\alpha^{FL}$ (250 ng)	1:5	500	800
			1000	300

Table 5 Anti-RAS Macromolecules 96-Well Plate Layout for EGF Treatment

	1	2	3	4	5	6	7	8	9	10	11	12
A B C D		No competitor	Competitor 1 100 ng	Competitor 1 500 ng	Competitor 1 1000 ng	No competitor	Competitor 2 100 ng	Competitor 2 500 ng	Competitor 2 1000 ng	Non-treated EGF rows		
E F G H		No competitor	Competitor 1 100 ng	Competitor 1 500 ng	Competitor 1 1000 ng	No competitor	Competitor 2 100 ng	Competitor 2 500 ng	Competitor 2 1000 ng	EGF treated rows		

Table 6 Ratio of DNA Used for the Competition Assays with Small Molecules

RLuc8 constructs (DNA ng)	GFP^2 constructs (DNA ng)	RLuc8:GFP^2 constructs ratio
KRAS (50 ng)	PI3Kα RBD (150 ng)	1:3
	PI3Kγ RBD (100 ng)	1:1
	CRAF RBD (100 ng)	1:1
	RALGDS RA (100 ng)	1:1
	$CRAF^{FL}$ (100 ng)	1:2
	$PI3K\alpha^{FL}$ (250 ng)	1:5
NRAS (50 ng)	PI3Kα RBD (150 ng)	1:3
	PI3Kγ RBD (100 ng)	1:1
NRAS (40 ng)	CRAF RBD (20 ng)	1:0.5
	RALGDS RA (20 ng)	1:0.5
NRAS (50 ng)	$CRAF^{FL}$ (100 ng)	1:2
HRAS (40 ng)	PI3Kα RBD (120 ng)	1:3
	PI3Kγ RBD (40 ng)	1:1
	CRAF RBD (20 ng)	1:0.5
	RALGDS RA (20 ng)	1:0.5
	$CRAF^{FL}$ (80 ng)	1:2

Table 7 Volume of DMSO and Compound to Add to Make a 10× Intermediate Compound Solution in 200 μl, 2% DMSO

Final concentration (μM)	0	50	100	200
DMSO (μl)	4	3	2	0
10 mM compound in 100% DMSO (μl)	0	1	2	4
OptiMEM no phenol red + 4% FBS (μl)			196	

Table 8 Compound 96-Well Plate Layout

<table>
<tr><th></th><th>1</th><th>2</th><th>3</th><th>4</th><th>5</th><th>6</th><th>7</th><th>8</th><th>9</th><th>10</th><th>11</th><th>12</th></tr>
<tr><td>A</td><td rowspan="4"></td><td rowspan="4">DMSO</td><td rowspan="4">Compound 1
5 μM</td><td rowspan="4">Compound 1
10 μM</td><td rowspan="4">Compound 1
20 μM</td><td rowspan="4">DMSO</td><td rowspan="4">Compound 2
5 μM</td><td rowspan="4">Compound 2
10 μM</td><td rowspan="4">Compound 2
20 μM</td><td rowspan="4"></td><td rowspan="4"></td><td rowspan="4"></td></tr>
<tr><td>B</td></tr>
<tr><td>C</td></tr>
<tr><td>D</td></tr>
<tr><td>E</td><td rowspan="4"></td><td rowspan="4">DMSO</td><td rowspan="4">Compound 3
5 μM</td><td rowspan="4">Compound 3
10 μM</td><td rowspan="4">Compound 3
20 μM</td><td rowspan="4">DMSO</td><td rowspan="4">Compound 4
5 μM</td><td rowspan="4">Compound 4
10 μM</td><td rowspan="4">Compound 4
20 μM</td><td rowspan="4"></td><td rowspan="4"></td><td rowspan="4"></td></tr>
<tr><td>F</td></tr>
<tr><td>G</td></tr>
<tr><td>H</td></tr>
</table>

Table 9 Compound 96-Well Plate Layout for EGF Treatment

<table>
<tr><th></th><th>1</th><th>2</th><th>3</th><th>4</th><th>5</th><th>6</th><th>7</th><th>8</th><th>9</th><th>10</th><th>11</th><th>12</th></tr>
<tr><td>A</td><td rowspan="4"></td><td rowspan="4">DMSO</td><td rowspan="4">Compound 1
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10 μM</td><td rowspan="4">Compound 1
20 μM</td><td rowspan="4">DMSO</td><td rowspan="4">Compound 2
5 μM</td><td rowspan="4">Compound 2
10 μM</td><td rowspan="4">Compound 2
20 μM</td><td rowspan="4" colspan="3">Non-treated EGF rows</td></tr>
<tr><td>B</td></tr>
<tr><td>C</td></tr>
<tr><td>D</td></tr>
<tr><td>E</td><td rowspan="4"></td><td rowspan="4">DMSO</td><td rowspan="4">Compound 1
5 μM</td><td rowspan="4">Compound 1
10 μM</td><td rowspan="4">Compound 1
20 μM</td><td rowspan="4">DMSO</td><td rowspan="4">Compound 2
5 μM</td><td rowspan="4">Compound 2
10 μM</td><td rowspan="4">Compound 2
20 μM</td><td rowspan="4" colspan="3">EGF treated rows</td></tr>
<tr><td>F</td></tr>
<tr><td>G</td></tr>
<tr><td>H</td></tr>
</table>

Table 10 Ratio of DNA Used for the Competition Assays with Small Molecules (Short Incubation)

RLuc8 constructs (DNA ng)	GFP^2 constructs (DNA ng)	RLuc8:GFP^2 constructs ratio
KRAS (50 ng)	$CRAF^{FL}$ (100 ng)	1:1
	$PI3K\alpha^{FL}$ (250 ng)	1:5

Table 11 Key Troubleshooting Steps

Step	Problem	Possible reasons	Advice
Basic Protocol 1	BRET pair of proteins of interest (POI) are not functioning correctly	GFP^2/RLuc8 reporter moieties are interfering with protein function	Add the reporter moieties to the other end of the protein or increase the linker length between the protein of interest and the moiety
		There was an error in construct generation	Sequence and check entire fusion protein cDNA
BRET measurement	Low luminescence or fluorescence detected by the instrument	The donor and/or the acceptor constructs are poorly expressed or interact weakly or sub-cellular location incorrect of one or both of the POI	Change the transfection reagent, try a codon optimization, check the localization of the POI by immunofluorescence
		Substrate is not working	Check the storage condition and try to use another aliquot
		Substrate was not added	Add the substrate
			Check the injectors (if used)
		Instrument is not working	Check the BRET configuration/protocol on the instrument
BRET measurement	Luminescence and fluorescence signals are produced but no BRET signal detected despite validated BRET pair proteins	Distance between the donor and acceptor molecules is too high	Change the position of the donor and acceptor moieties on the POI
		The orientation of the donor and/or acceptor moieties is not optimal for BRET	Increase the linker length between the POI and the GFP^2/RLuc8
Basic Protocol 3, Alternate Protocols 2 and 3	No decrease of the BRET signal upon inhibitor treatment	Inhibitor not working in cells	Include a positive inhibitor as control
		Inhibitor interfering with the assay	Check the RLuc8 and GFP^2 channels
			Add a negative control BRET pair
		Too high expression of the donor and/or acceptor plasmids inducing a titration of the inhibitor	Decrease the donor/acceptor ratio