ThermoFisher SCIENTIFIC

Screening Protocol and Assay Conditions

Revised 07-07-2023

Overview and Assay Theory

The SelectScreen Cell-based GPCR Profiling (SSCG) Service utilizes Life Technologies' growing library of functional and validated GeneBLAzer™ and Tango™ GPCR cell lines. Both of these GPCR signaling technologies, discussed in detail below, activate a stably integrated beta-lactamase (*bla*) reporter gene. When present, the *bla* enzyme cleaves the LiveBLAzer™-FRET B/G Loading Substrate to provide a selective and quantitative FRET-based readout of GPCR activity. The rapidly growing panel of targets available in the Service is available in two different formats, division arrested or cryo-preserved. While not a format included in our standard service options, dividing cells are sometimes utilized in the profiling service. Targets requiring the use of dividing cells within the service may be identified within the Cell Line-Specific Assay Conditions. The Service provides a reliable, rapid, and sensitive method of analyzing the status of a wide range of disease-relevant GPCRs upon exposure to drug candidates or other stimuli.

All GeneBLAzer and Tango GPCR cell lines used in the SelectScreen Cell-based GPCR Profiling (SSCG) Service provide superior response profiles as a result of optimization of assay conditions and selection of high-responding cell populations. Since the cell lines in the portfolio have been developed and extensively validated by Life Technologies, we ensure the consistency, reliability and performance of each cell line. Each of the GeneBLAzer and Tango GPCR cell lines:

Are division arrested or cryo-preserved to avoid variability associated with cell division

Provide ready-to-screen, ratiometric assays for disease relevant targets

Are functionally validated to ensure high-quality results each and every time

In order to evaluate non-specific effects when using GeneBLAzer GPCR technology, parental cell lines are included in the panel to allow counter-screening of the identified "hits" from the primary screen.

The GeneBLAzer and Tango cell lines in the Service are tested and documented to show a high level of performance. These assays have been designed and validated to meet the following specifications:

Z′-factor of 0.5 or greater for Agonist assays and Z′-factor of 0.4 or greater for Antagonist assays.

Appropriate EC_{50}/IC_{50} responses to known Agonists and Antagonists

Any assay results not meeting these specifications are automatically repeated until the results pass our QC criteria.

How GeneBLAzer and Tango Technology works

GeneBLAzer and Tango Technology use a mammalian-optimized Beta-lactamase reporter gene (*bla*) combined with a FRET-enabled substrate to provide reliable and sensitive detection in intact cells.

Cells are loaded with an engineered fluorescent substrate containing two fluorophores, coumarin and fluorescein. In the absence of *bla* expression, the substrate molecule remains intact. In this state, excitation of the coumarin results in fluorescence resonance energy transfer to the fluorescein moiety and emission of green light. However, when *bla* is expressed, the substrate is cleaved, separating the fluorophores, and disrupting energy transfer. Excitation of the coumarin in the presence of Beta-lactamase (*bla*) activity results in a blue fluorescence signal. The resulting coumarin:fluorescein ratio provides a normalized reporter response which can minimize experimental noise that can mask the underlying biological response of interest.

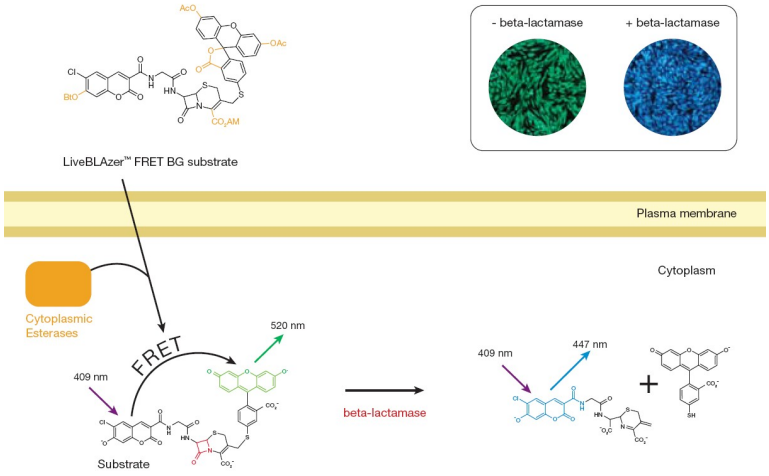

Figure 1. Fluorescent detection of cells using the GeneBLAzer and Tango™ technology. After substrate loading, in the absence of Beta-lactamase, cells appear green. In the presence of Beta-lactamase, the substrate is cleaved and cells therefore appear blue.

Detecting GPCR signaling using the GeneBLAzer Technology

GPCR signaling as detected by the GeneBLAzer Technology is mediated by trimeric G-proteins containing alpha, beta, and gamma subunits and can be categorized into signaling classes based upon the alpha-subunit composition. G_s, G_q, $G_{i/o}$ proteins mediate intracellular signaling through activation of signaling pathways leading to distinct physiological endpoints. Activation of G_s and $G_{i/o}$ coupled receptors leads to stimulation or inhibition of adenylate cyclase, respectively, while activation of G_q coupled receptors results in stimulation of phospholipase C. However, co-transfection with the promiscuous or chimeric G_{a15}, G_{qi5}, or G_{qo5} G- proteins redirects this signaling through PLC. GPCR signaling through these distinct pathways thus can be monitored by modulation of specific transcriptional response elements placed upstream of the *bla*.

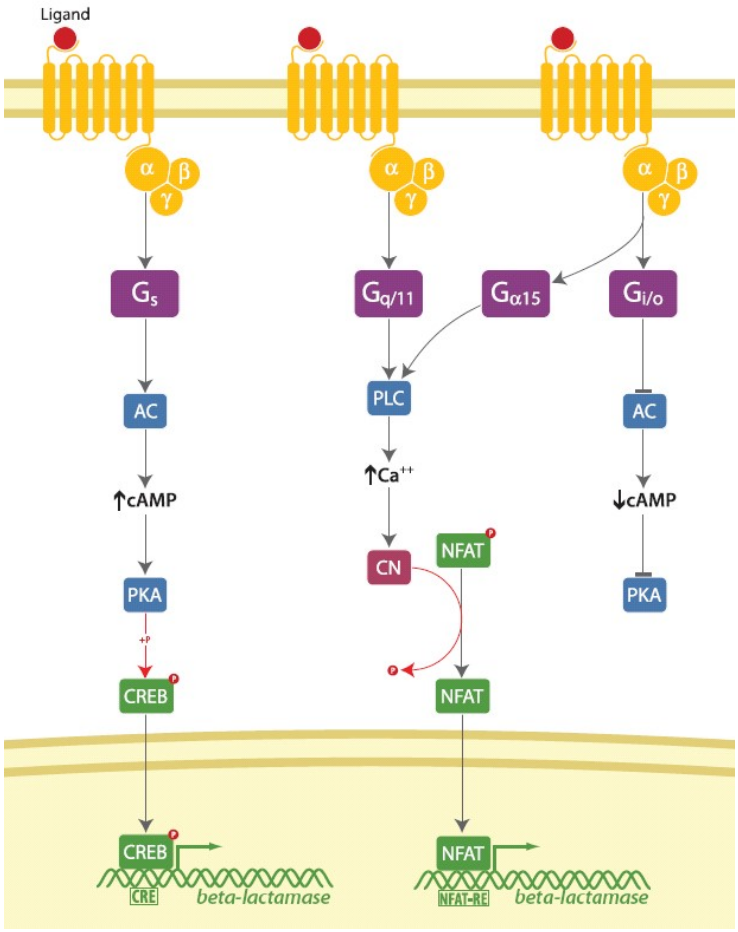

Figure 2. GPCR activity using GeneBLAzer validated assays. Schematic overview of the activation of GPCRs through distinct pathways and analysis of the response through the Beta-lactamase reporter gene system.

Detecting GPCR signaling using the Tango Technology

GPCR signaling as detected by the Tango Technology is based upon the interaction of intracellular Beta-arrestin proteins and the target receptor. Upon ligand binding to the target receptor, the protease tagged arrestin is stimulated and recruited to the protease site on the C-terminus of the GPCR, which triggers release of the tethered transcription factor. The free transcription factor then enters the nucleus and stimulates the *bla* activity.

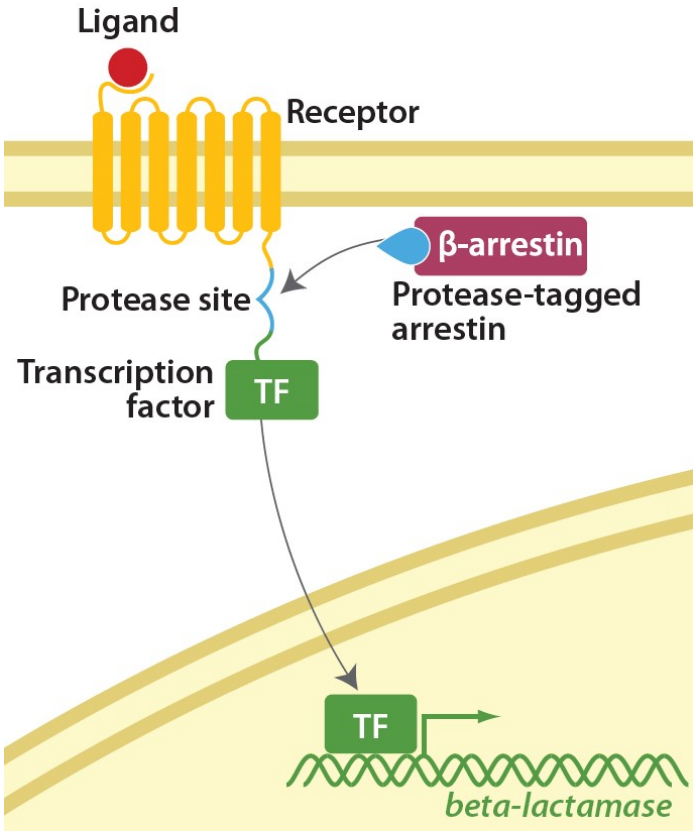

Figure 3. GPCR activity using Tango™ validated assays. Schematic overview of the activation of GPCRs through Beta-arrestin signaling pathway and analysis of the response through the Beta-lactamase reporter gene system.

SelectScreen ASSAY CONDITIONS

Test Compounds

Test compounds are received at 1000X (or greater) of the desired starting concentration in 100% DMSO. If compounds are supplied at greater than 1000X concentration, an initial dilution is made in 100% DMSO to bring the compounds to 1000X concentration. The 1000X test compounds are serially diluted (10 point ½-log increments) in 100% DMSO.

Substrate Loading Solution

The Substrate Loading Solution consists of three reagents: Solution A (1 mM LiveBLAzer™-FRET B/G Substrate); Solution B, and Solution C.

Agonist Assay Protocol

Plate type utilized and the addition of cells (Step 1) or compound (Step 2) first to the plate is dictated by each cell line and described in the Cell Line-Specific Assay Conditions.

Barcoded Corning 384 well Flat Clear Bottom Black Polystyrene TC-Treated Microplates (Corning Cat. #3712)

Barcoded Corning 384 well Flat Clear Bottom Black Polystyrene Poly-D-Lysine Coated Microplates (Corning Cat. #3664)

1. 32 µL of cells diluted in Assay Media to appropriate cell density are added to the assay plate. If needed, cells are incubated at 37°C/5% CO_2 for 6 or 16-24 hours (depending upon cell line specifics) before compound is added.
2. 40 nL of 1000X compound or known activator titration plus 4 µL of assay media is added to the cells in the assay plate.
3. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL.
4. The assay plate is incubated for 5 or 16 hours (depending upon cell line specifics) at 37ºC/5% CO_2 in a humidified incubator.
5. 8 µL of the Substrate Loading Solution is added to the assay plate.
6. The assay plate is incubated for 2 hours at room temperature, in the dark.
7. The assay plate is read on a fluorescence plate reader (Tecan Safire^2) and the data is analyzed.

Antagonist Assay Protocol

An Agonist assay screen is run to obtain the EC_{80} concentration of the known activator to add in step 3.

1. 32 µL of cells diluted in Assay Media to appropriate cell density are added to the assay plate. If needed, cells are incubated at 37°C/5% CO_2 for 6 or 16-24 hours (depending upon cell line specifics) before compound is added.
2. 40 nL of 1000X compound or known antagonist titration plus 4 µL of Assay Media is added to the cells in the assay plate and incubated for 30 minutes at 37°C/5% CO_2 in a humidified incubator.
3. 4 µL of the 10X EC_{80} concentration of agonist, as determined in an Agonist assay, is added to all wells containing test compound and known inhibitor to bring the final assay volume to 40 µL.
4. 4 µL of Assay Media is added to remaining control wells to bring the volume up to 40 µL.
5. The assay plate is incubated for 5 or 16 hours (depending upon cell line specifics) at 37ºC/5% CO_2 in a humidified incubator.
6. 8 µL of the Substrate Loading Solution is added to the assay plate.

7. The assay plate is incubated for 2 hours at room temperature, in the dark.
8. The assay plate is read on a fluorescence plate reader (Tecan Safire^2) and the data is analyzed.

SelectScreen Assay Controls

The following controls are run on each plate for each individual cell-line:

Full Agonist control

The full agonist control contains 0.1% DMSO, cells and a maximum concentration of the known agonist (stim). In agonist mode, the full agonist control is used to determine the upper end of the assay or 100% activation. In antagonist mode, the full agonist control is used to determine the actual EC_{80} used in the assay with the EC_{80} concentration chosen from previous agonist experiments.

No Agonist control

The no agonist control contains 0.1% DMSO, cells and assay media in place of the agonist (stim). In agonist mode, it is used to determine the lower end of the assay or 0% activation. In antagonist mode, it is used to determine maximal inhibition or 100% inhibition.

Cell-free Control

The cell-free control contains 0.1% DMSO and assay media. It is used to determine the background fluorescence for both coumarin and fluorescein wavelengths. This value is used for background subtraction.

EC_{80} Control (Antagonist mode only)

The EC_{80} control is a concentration of the known agonist in assay media that has been determined through an agonist experiment. In antagonist mode, the EC_{80} control is used to determine the actual baseline of activation or 0% inhibition.

Known Agonist (Agonist mode) or Antagonist (Antagonist mode) Titration

A known agonist or antagonist titration is run on every plate for each cell-line to ensure the cell line is either activated or inhibited within an expected EC_{50}/IC_{50} range as previously determined.

SelectScreen Data Analysis

The following equations are used for each set of data points:

	Equation
Background-Subtracted Fluorescence (Fl = Fluorescence Intensity)	$\mathbf{Fl}_{\text{Sample}} - \mathbf{Fl}_{\text{Cell-Free Ctrl}}$
Emission Ratio (using values corrected for background fluorescence)	$\dfrac{\textbf{Coumarin Emission (460 nm)}}{\textbf{Fluorescein Emission (530 nm)}}$
Response Ratio (Act. = Activation)	$\dfrac{\textbf{Emission Ratio}_{\text{Compound}}}{\textbf{Emission Ratio}_{\text{No Agonist Ctrl}}}$
% Activation – Agonist Assays	$\left\{ \dfrac{\textbf{Response Ratio}_{\text{Compound}} - \textbf{Response Ratio}_{\text{No Agonist Ctrl}}}{\textbf{Response Ratio}_{\text{Full Agonist Ctrl}} - \textbf{Response Ratio}_{\text{No Agonist Ctrl}}} \right\} * 100$
% Inhibition – Antagonist Assays	$\left\{ 1 - \dfrac{\textbf{Response Ratio}_{\text{Compound}} - \textbf{Response Ratio}_{\text{No Agonist Ctrl}}}{\textbf{Response Ratio}_{\text{EC80 Ctrl}} - \textbf{Response Ratio}_{\text{No Agonist Ctrl}}} \right\} * 100$
Z´ - Agonist Assays (using Emission Ratio values)	$1 - \dfrac{\textbf{3*Std Dev}_{\text{Full Agonist Ctrl}} + \textbf{3*Std Dev}_{\text{No Agonist Ctrl}}}{\textbf{Mean}_{\text{Full Agonist Ctrl}} - \textbf{Mean}_{\text{No Agonist Ctrl}}}$
Z´ - Antagonist Assays (using Emission Ratio values)	$1 - \dfrac{\textbf{3*Std Dev}_{\text{EC80 Ctrl}} + \textbf{3*Std Dev}_{\text{No Agonist Ctrl}}}{\textbf{Mean}_{\text{EC80 Ctrl}} - \textbf{Mean}_{\text{No Agonist Ctrl}}}$

Graphing Software

SelectScreen Cell-Based GPCR Profiling Service uses XL*fit* from IDBS. The dose response curve is curve fit to model number 205 (sigmoidal dose-response model). Custom logic was built in-house for the data analysis tool to address the different compound characteristics that can be observed with functional assays. Using this logic the relative EC_{50}/IC_{50} value for each given compound is provided.

GPCR Profiling Cell Lines Available for Screening

Assay	Cell Line	Tech-nology	Agonist	EC50 (nM)	Antagonist	IC50 (nM)	Ag. Mode	Antag. Mode
ADORA1	bla U2OS	Tango	NECA	16.4	DPCPX	5.61	Yes	Yes
ADORA3	bla U2OS	Tango	NECA	66.5	MRS1220	0.459	Yes	Yes
ADRA1A	NFAT-bla CHO-K1	BLA	Phenylephrine	43.4	Prazosin	1.58	Yes	Yes
ADRA1B	NFAT-bla CHO-K1	BLA	Phenylephrine	71.05	Yohimbine	1081	Yes	Yes
ADRA2A	bla U2OS	Tango	UK 14304	4.04	Yohimbine	38.7	Yes	Yes
ADRA2A	Gqo5-NFAT-bla CHO-K1	BLA	UK 14304	11.5	Yohimbine	14.2	Yes	Yes
ADRB1	CRE-bla CHO-K1	BLA	(-)Denopamine	35.3	CPG-20712A	1.24	Yes	Yes
ADRB2	CRE-bla CHO-K1	BLA	Isoproterenol	3.896	Alprenolol	0.287	Yes	Yes
ADRB3	CRE-bla CHO-K1	BLA	BRL 37344	4.61	ICI-118551	4,460	Yes	Yes
AGTR1	bla U2OS	Tango	Angiotensin II	2.03	Telmisartan	0.025	Yes	Yes
AGTRL1	bla U2OS	Tango	Apellin-13	2.10	None	---	Yes	Yes
AVPR1A	NFAT-bla CHO-K1	BLA	dAVP	0.864	d(CH2)5(Tyr(Me)2)AVP	2.23	Yes	Yes
AVPR2	CRE-bla CHO-K1	BLA	dDAVP	0.501	d(CH2)5(Tyr(Et)2)AVP	111.3	Yes	Yes
B1	NFAT-bla CHO-K1	BLA	Bradykinin	17.1	Bradykinin (D-Arg-O Hyp3 Igl5 D-Igl7 Oic8)	128	Yes	Yes
B2	NFAT-bla CHO-K1	BLA	Bradykinin Acetate	1.72	HOE-140	73.8	Yes	Yes
C5AR1	Galpha15-NFAT-bla CHO-K1	BLA	C5a	5.16	None	---	Yes	Yes
CALCR	CRE-bla Freestyle 293F	BLA	sCT	0.003	sCT(8-32)	8.03	Yes	Yes
CALCRL:RAMP1	CRE-bla Freestyle 293F	BLA	CGRP	0.154	CGRP(8-37)	232	Yes	Yes
CALCRL:RAMP3	CRE-bla Freestyle 293F	BLA	Adrenomedullin	0.348	None	---	Yes	Yes
CaSR	Gqo5-NFAT-bla CHO-K1	BLA	Spermine	274,000	None	---	Yes	Yes
CCKAR	NFAT-bla HEK 293T	BLA	CCK-8	0.471	Lorglumide	360	Yes	Yes
CCKBR	NFAT-bla HEK 293T	BLA	CCK-8	4.457	None	---	Yes	Yes
CCR1	bla U2OS	Tango	Mip1-alpha	1.66	J113863	5.777	Yes	Yes
CCR2	bla U2OS	Tango	MCP-1	1.10	BMS CCR2 22	11.65	Yes	Yes
CCR3	bla U2OS	Tango	Eotaxin	72.2	None	---	Yes	Yes
CCR4	bla U2OS	Tango	MDC	11.2	None	---	Yes	Yes
CCR5	bla U2OS	Tango	Mip1-alpha	10.6	Maraviroc	0.087	Yes	Yes
CCR6	bla U2OS	Tango	Mip3-alpha	0.092	None	---	Yes	Yes
CCR7	bla U2OS	Tango	Mip3-beta	77.1	None	---	Yes	Yes
CMKLR1	bla U2OS	Tango	Chemerin	1.19	None	---	Yes	Yes
CNR1	Galpha15-NFAT-bla CHO-K1	BLA	CP-55940	4.66	AM251	2.349	Yes	Yes
CNR2	bla U2OS	Tango	CP-55940	30.5	None	---	Yes	Yes
CRHR1	CRE-bla CHO-K1	BLA	CRF	0.042	Astressin	1.535	Yes	Yes
CRHR2	CRE-bla CHO-K1	BLA	CRF	0.030	Astressin	0.849	Yes	Yes

CXCR1	bla U2OS	Tango	IL-8	3.31	None	---	Yes	Yes
CXCR2	bla U2OS	Tango	IL-8	4.07	None	---	Yes	Yes
CXCR3	bla U2OS	Tango	I-TAC	7.26	None	---	Yes	Yes
CXCR4	bla U2OS	Tango	SDF1-alpha	12.6	None	---	Yes	Yes
CXCR6	bla U2OS	Tango	CXCL16	2.61	None	---	Yes	Yes
CXCR7	bla U2OS	Tango	SDF1-alpha	1.12	None	---	Yes	Yes
cysLT2	NFAT-bla CHO-K1	BLA	LTD4	51.3	None	---	Yes	Yes
D1	CRE-bla CHO-K1	BLA	Dihydrexidine	11.7	R(+)-SCH-23390	137.8	Yes	Yes
D2	Gqo5-NFAT-bla CHO-K1	BLA	Apomorphine	132.5	Perphenazine	0.480	Yes	Yes
EDG1	bla U2OS	Tango	S1P	0.987	VPC23019	47.3	Yes	Yes
EDG2	bla U2OS	Tango	LPA (18:1)	4,640	Ki16425	65.0	Yes	Yes
EDG3	Galpha15-NFAT-bla HEK 293T	BLA	S1P	274	CAY10444	23,700	Yes	Yes
EDG4	bla U2OS	Tango	LPA (18:1)	6830	None	---	Yes	Yes
EDG6	bla U2OS	Tango	S1P	168	None	---	Yes	Yes
EDG7	NFAT-bla HEK 293T	BLA	LPA (18:1)	956	VPC32183	3,020	Yes	Yes
EDG8	bla U2OS	Tango	S1P	85.8	None	---	Yes	Yes
EDNRA	bla U2OS	Tango	ET-1	0.017	BQ-123	1,090	Yes	Yes
EDNRA	NFAT-bla HEK 293T	BLA	ET-1	0.112	BQ-123	3,189	Yes	Yes
EDNRB	NFAT-bla HEK 293T	BLA	ET-1	0.149	BQ-788	572.8	Yes	Yes
F2RL1	bla U2OS	Tango	SLIGRL-NH2	365	None	---	Yes	Yes
FPRL1	Galpha15-NFAT-bla CHO-K1	BLA	WKYMVM peptide	2.22	None	---	Yes	Yes
GALR1	bla U2OS	Tango	Galanin	77.2	None	---	Yes	Yes
GALR2	bla U2OS	Tango	Galanin (1-30)	11.1	None	---	Yes	Yes
GCGR	CRE-bla CHO-K1	BLA	Glucagon	0.1734	None	---	Yes	Yes
GHSR	TREx-bla U2OS	Tango	None		Substance P	94.4		Yes
GIPR	CRE-bla HEK 293T	BLA	Gastric Inhibitory Peptide	0.015	None	---	Yes	Yes
GLP1R	CRE-bla CHO-K1	BLA	GLP-1	5.129	Excendin-3	118	Yes	Yes
GLP2R	bla U2OS	Tango	GLP-2	4.42	None	---	Yes	Yes
GnRHR	NFAT-bla CHO-K1	BLA	LH-RH	0.660	LH-RH antagonist	1,730	Yes	Yes
GPR1	bla U2OS	Tango	Chemerin	0.127	None	---	Yes	Yes
GPR10	NFAT-bla CHO-K1	BLA	PrRP20	0.051	None	---	Yes	Yes
GPR109A	bla U2OS	Tango	Nicotinic Acid	13,000	None	---	Yes	Yes
GPR119	TREx-CRE-bla CHO-K1	BLA	AR231453	4.67	None	---	Yes	Yes
GPR35	bla U2OS	Tango	Zaprinast	1,930	None	---	Yes	Yes
GPR44 (CRTH2)	bla U2OS	Tango	Indomethicin	875	BAY-u3405	19,300	Yes	Yes
GPR54	NFAT-bla CHO-K1	BLA	Metastin (45-54)	3.65	None	---	Yes	Yes
GPR8	bla U2OS	Tango	Neuropeptide B-29	27.8	None	---	Yes	Yes

GRPR	NFAT-bla CHO-K1	BLA	GRP	0.007	None	---	Yes	Yes
H1	NFAT-bla HEK 293T	BLA	Histamine	8.95	Triprolidine	9.82	Yes	Yes
H2	CRE-bla HEK 293T	BLA	Histamine	7.24	Tiotidine	71.8	Yes	Yes
H3	bla U2OS	Tango	Methylhistamine	31.8	Thioperamide	481.5	Yes	Yes
H4	bla U2OS	Tango	4-Methylhistamine	60.1	Thioperamide	385.6	Yes	Yes
HCRTR1	NFAT-bla CHO-K1	BLA	Orexin A	0.493	SB 408124	67.0	Yes	Yes
HCRTR2	Galpha15-NFAT-bla CHO-K1	BLA	Orexin B	1.48	EMPA	46.12	Yes	Yes
HTR1A	bla U2OS	Tango	5-HT	15.62	WAY-100635	435.8	Yes	Yes
HTR1A	Galpha15-NFAT-bla CHO-K1	BLA	5-HT	336	Methiothepin	35.5	Yes	Yes
HTR2A	bla U2OS	Tango	5-HT	5.497	Mianserin	68.4	Yes	Yes
HTR2B	NFAT-bla CHO-K1	BLA	5-HT	2.27	Clozapine	9.012	Yes	Yes
HTR7	CRE-bla CHO-K1	BLA	5-HT	8.38	SB269970	1.949	Yes	Yes
LTB4R	Galpha15-NFAT-bla CHO-K1	BLA	Leukotriene B4	58.4	None	---	Yes	Yes
M1	NFAT-bla CHO-K1	BLA	Carbachol	151	Scopolamine	4.50	Yes	Yes
M2	bla U2OS	Tango	Carbachol	5,691	Scopolamine	26.6	Yes	Yes
M3	NFAT-bla CHO-K1	BLA	Carbachol	172	Scopolamine	4.725	Yes	Yes
M4	bla U2OS	Tango	Carbachol	3,040	Scopolamine	0.392	Yes	Yes
M4	Gqo5-NFAT-bla CHO-K1	BLA	Carbachol	547	DAMP	3.01	Yes	Yes
M5	NFAT-bla CHO-K1	BLA	Carbachol	489	Scopolamine	3.22	Yes	Yes
MC1R	CRE-bla CHO-K1	BLA	NDP-alpha-MSH	0.173	None	---	Yes	Yes
MC2R	CRE-bla CHO-K1	BLA	ACTH (1-24)	0.046	None	---	Yes	Yes
MC3R	CRE-bla CHO-K1	BLA	NDP-alpha-MSH	0.034	SHU9119	1.03	Yes	Yes
MC4R	CRE-bla CHO-K1	BLA	alpha-MSH	0.645	SHU9119	0.041	Yes	Yes
MC5R	CRE-bla CHO-K1	BLA	NDP-alpha-MSH	0.140	None	---	Yes	Yes
MCHR1 (GPR24)	Gqi5-NFAT-bla CHO-K1	BLA	MCH	7.76	ATC0175	0.961	Yes	Yes
MCHR2	bla U2OS	Tango	MCH (6-17)	92.1	None	---	Yes	Yes
MCHR2	NFAT-bla CHO-K1	BLA	MCH (6-17)	0.973	None	---	Yes	Yes
MTNR1A	bla U2OS	Tango	Melatonin	0.713	Luzindole	1,940	Yes	Yes
MTNR1B	bla U2OS	Tango	Melatonin	83.6	None	---	Yes	Yes
NMUR1	NFAT-bla CHO-K1	BLA	NMU-25	0.051	None	---	Yes	Yes
NPSR1-A	NFAT-bla CHO-K1	BLA	Neuropeptide S	2.00	None	---	Yes	Yes
NPSR1-B	NFAT-bla CHO-K1	BLA	Neuropeptide S	11.2	None	---	Yes	Yes
NPY1R	bla U2OS	Tango	Neuropeptide Y	0.251	GR231118	7.19	Yes	Yes
NPY2R	bla U2OS	Tango	Neuropeptide Y	16.1	BIIE 0246	2.48	Yes	Yes
NTSR1	NFAT-bla CHO-K1	BLA	Neurotensin	0.307	None	---	Yes	Yes
OPRD1	bla U2OS	Tango	SNC80	97.2	SDM25N	9.10	Yes	Yes
OPRK1	bla U2OS	Tango	U50488	4.39	nor-BNI	1.26	Yes	Yes

OPRK1	Gqo5-NFAT-bla CHO-K1	BLA	U50488	13.2	nor-BNI	1.26	Yes	Yes
OPRL1	bla U2OS	Tango	Nociceptin	4.92	UFP-101	27.8	Yes	Yes
OPRL1	Gqi5-NFAT-bla Freestyle 293F	BLA	Nociceptin	1.62	UFP-101	67.0	Yes	Yes
OPRM1	bla U2OS	Tango	DAMGO	19.968	beta-funaltrexamine	5.666	Yes	Yes
OXTR	Gqo5-NFAT-bla CHO-K1	BLA	Oxytocin	31.9	Atosiban	525.1	Yes	Yes
P2RY11	NFAT-bla CHO-K1	BLA	ATP	1,490	Suramin	13,500	Yes	Yes
P2RY2	NFAT-bla CHO-K1	BLA	ATP	677	Suramin	209,000	Yes	Yes
P2RY6	NFAT-bla CHO-K1	BLA	UDP	22.5	Suramin	71,300	Yes	Yes
PAC1	CRE-bla CHO-K1	BLA	PACAP	0.002	None	---	Yes	Yes
PTAFR	bla U2OS	Tango	PAF	14.47	ABT-491	0.024	Yes	Yes
PTGDR	CRE-bla CHO-K1	BLA	PGD2	1.46	AH6809	4494	Yes	Yes
PTGER1	NFAT-bla CHO-K1	BLA	PGE2	4.44	AH6809	1,560	Yes	Yes
PTGER2	CRE-bla CHO-K1	BLA	PGE2	1.57	AH6809	1,540	Yes	Yes
PTGIR	CRE-bla CHO-K1	BLA	Iloprost	0.011	CAY10441	1.20	Yes	Yes
RLN3R1	bla U2OS	Tango	Relaxin-3	6.28	None	---	Yes	Yes
SCTR	CRE-bla CHO-K1	BLA	Secretin	0.003	None	---	Yes	Yes
SSTR1	bla U2OS	Tango	SST14	6.48	None	---	Yes	Yes
SSTR2	bla U2OS	Tango	SST14	0.191	CYN154806	39.3	Yes	Yes
SSTR5	bla U2OS	Tango	SST14	2.00	None	---	Yes	Yes
TACR1	bla U2OS	Tango	Sar9 Met(O2)11 Substance P	1.92	None	---	Yes	Yes
TACR2	NFAT-bla CHO-K1	BLA	(Nle10)-Neurokinin A (4-10)	1.30	MEN-10376	92.1	Yes	Yes
TACR3	bla U2OS	Tango	SSP Frag 6-11	0.780	None	---	Yes	Yes
TBXA2R	bla U2OS	Tango	U46619	123	L655240	21.4	Yes	Yes
VPAC1	CRE-bla CHO-K1	BLA	VIP	0.040	VIP1 Antagonist	2.01	Yes	Yes
VPAC2	CRE-bla CHO-K1	BLA	VIP	0.043	None	---	Yes	Yes

*EC_{50} and IC_{50} values are representative

Parental Cell Lines Available for Screening

Cell Line	Tech-nology	Agonist	EC50 (nM)
CRE-bla CHO-K1	BLA	Forskolin	105
CRE-bla Freestyle 293F	BLA	Forskolin	1,500
CRE-bla HEK 293T	BLA	Forskolin	301
Galpha15-NFAT-bla CHO-K1	BLA	Thapsigargin	2.75
Galpha15-NFAT-bla HEK 293T	BLA	Thapsigargin + 5 nM PMA	1.41
Gqi5-NFAT-bla Freestyle 293F	BLA	PMA	1.42
Gqo5-NFAT-bla CHO-K1	BLA	Thapsigargin	3.16
NFAT-bla CHO-K1	BLA	Thapsigargin	3.69
NFAT-bla HEK 293T	BLA	Thapsigargin + 5 nM PMA	1.19

*EC_{50} values are representative

Cell Line-Specific Assay Conditions

ADORA1 - bla U2OS - Agonist Screen
ADORA1-bla U2OS cells are thawed and resuspended in Assay Media (Freestyle media) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of NECA (control agonist starting concentration, 500 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

ADORA1 - bla U2OS - Antagonist Screen, Activated by NECA
ADORA1-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of DPCPX (control antagonist starting concentration, 5,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist NECA at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

ADORA3 - bla U2OS - Agonist Screen
ADORA3-bla U2OS cells are thawed and resuspended in Assay Media (Freestyle media) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of NECA (control agonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

ADORA3 - bla U2OS - Antagonist Screen, Activated by NECA
ADORA3-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of MRS1220 (control antagonist starting concentration, 100 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist NECA at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

ADRA1A - NFAT-bla CHO-K1 - Agonist Screen
ADRA1A-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Phenylephrine (control agonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

ADRA1A - NFAT-bla CHO-K1 - Antagonist Screen, Activated by Phenylephrine
ADRA1A-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Prazosin (control antagonist starting concentration, 20,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Phenylephrine at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

ADRA1B - NFAT-bla CHO-K1 - Agonist Screen
ADRA1B-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Phenylephrine (control agonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

ADRA1B - NFAT-bla CHO-K1 - Antagonist Screen, Activated by Phenylephrine
ADRA1B-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Yohimbine (control antagonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Phenylephrine at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

ADRA2A - bla U2OS - Agonist Screen
ADRA2A-bla U2OS cells are thawed and resuspended in Assay Media (Freestyle media) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of UK 14304 (control agonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

ADRA2A - bla U2OS - Antagonist Screen, Activated by UK 14304
ADRA2A-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Yohimbine (control antagonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist UK 14304 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

ADRA2A - Gqo5-NFAT-bla CHO-K1 - Agonist Screen
ADRA2A-Gqo5-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of UK 14304 (control agonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

ADRA2A - Gqo5-NFAT-bla CHO-K1 - Antagonist Screen, Activated by UK 14304
ADRA2A-Gqo5-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Yohimbine (control antagonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist UK 14304 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

ADRB1 - CRE-bla CHO-K1 - Agonist Screen
ADRB1-CRE-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of (-)Denopamine (control agonist starting concentration, 20,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

ADRB1 - CRE-bla CHO-K1 - Antagonist Screen, Activated by (-)Denopamine
ADRB1-CRE-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of CPG-20712A (control antagonist starting concentration, 100,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist (-)Denopamine at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

ADRB2 - CRE-bla CHO-K1 - Agonist Screen
ADRB2-CRE-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Isoproterenol (control agonist starting concentration, 500 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 2 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

ADRB2 - CRE-bla CHO-K1 - Antagonist Screen, Activated by Isoproterenol
ADRB2-CRE-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Alprenolol (control antagonist starting concentration, 100 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Isoproterenol at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 2 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

ADRB3 - CRE-bla CHO-K1 - Agonist Screen
ADRB3-CRE-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of BRL 37344 (control agonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

ADRB3 - CRE-bla CHO-K1 - Antagonist Screen, Activated by BRL 37344
ADRB3-CRE-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of ICI-118551 (control antagonist starting concentration, 1,000,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist BRL 37344 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

AGTR1 - bla U2OS - Agonist Screen
AGTR1-bla U2OS cells are thawed and resuspended in Assay Media (Freestyle media) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Angiotensin II (control agonist starting concentration, 10 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

AGTR1 - bla U2OS - Antagonist Screen, Activated by Angiotensin II
AGTR1-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Telmisartan (control antagonist starting concentration, 10 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Angiotensin II at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

AGTRL1 - bla U2OS - Agonist Screen
AGTRL1-bla U2OS cells are thawed and resuspended in Assay Media (Freestyle media) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Apellin-13 (control agonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

AGTRL1 - bla U2OS - Antagonist Screen, Activated by Apellin-13
AGTRL1-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Apellin-13 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the AGTRL1-bla U2OS assay does not have an antagonist control.

AVPR1A - NFAT-bla CHO-K1 - Agonist Screen
AVPR1A-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 156,250 cells/mL. 32 µL of cell suspension (5,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of dAVP (control agonist starting concentration, 100 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

AVPR1A - NFAT-bla CHO-K1 - Antagonist Screen, Activated by dAVP
AVPR1A-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of d(CH2)5(Tyr(Me)2)AVP (control antagonist starting concentration, 100 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist dAVP at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

AVPR2 - CRE-bla CHO-K1 - Agonist Screen
AVPR2-CRE-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 625,000 cells/mL. 32 µL of cell suspension (20,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of dDAVP (control agonist starting concentration, 30 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

AVPR2 - CRE-bla CHO-K1 - Antagonist Screen, Activated by dDAVP
AVPR2-CRE-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of d(CH2)5(Tyr(Et)2)AVP (control antagonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist dDAVP at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

B1 - NFAT-bla CHO-K1 - Agonist Screen
B1-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Bradykinin (control agonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

B1 - NFAT-bla CHO-K1 - Antagonist Screen, Activated by Bradykinin
B1-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Bradykinin (D-Arg-O Hyp3 Igl5 D-Igl7 Oic8) (control antagonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Bradykinin at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

B2 - NFAT-bla CHO-K1 - Agonist Screen
B2-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Bradykinin Acetate (control agonist starting concentration, 5,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

B2 - NFAT-bla CHO-K1 - Antagonist Screen, Activated by Bradykinin Acetate
B2-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of HOE-140 (control antagonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Bradykinin Acetate at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

C5AR1 - Galpha15-NFAT-bla CHO-K1 - Agonist Screen
C5AR1-Galpha15-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of C5a (control agonist starting concentration, 241 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

C5AR1 - Galpha15-NFAT-bla CHO-K1 - Antagonist Screen, Activated by C5a
C5AR1-Galpha15-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist C5a at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the C5AR1-Galpha15-NFAT-bla CHO-K1 assay does not have an antagonist control.

CALCR - CRE-bla Freestyle 293F - Agonist Screen
CALCR-CRE-bla Freestyle 293F cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well Poly-D-Lysine assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of sCT (control agonist starting concentration, 1 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CALCR - CRE-bla Freestyle 293F - Antagonist Screen, Activated by sCT
CALCR-CRE-bla Freestyle 293F cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well Poly-D-Lysine assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of sCT(8-32) (control antagonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist sCT at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CALCRL:RAMP1 - CRE-bla Freestyle 293F - Agonist Screen
CALCRL:RAMP1-CRE-bla Freestyle 293F cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well Poly-D-Lysine assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of CGRP (control agonist starting concentration, 100 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CALCRL:RAMP1 - CRE-bla Freestyle 293F - Antagonist Screen, Activated by CGRP
CALCRL:RAMP1-CRE-bla Freestyle 293F cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well Poly-D-Lysine assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of CGRP(8-37) (control antagonist starting concentration, 3,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist CGRP at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CALCRL:RAMP3 - CRE-bla Freestyle 293F - Agonist Screen
CALCRL:RAMP3-CRE-bla Freestyle 293F cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well Poly-D-Lysine assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Adrenomedullin (control agonist starting concentration, 100 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CALCRL:RAMP3 - CRE-bla Freestyle 293F - Antagonist Screen, Activated by Adrenomedullin
CALCRL:RAMP3-CRE-bla Freestyle 293F cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well Poly-D-Lysine assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Adrenomedullin at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the CALCRL:RAMP3-CRE-bla Freestyle 293F assay does not have an antagonist control.

CaSR - Gqo5-NFAT-bla CHO-K1 - Agonist Screen
CaSR-Gqo5-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Spermine (control agonist starting concentration, 1,000,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CaSR - Gqo5-NFAT-bla CHO-K1 - Antagonist Screen, Activated by Spermine
CaSR-Gqo5-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Spermine at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the CaSR-Gqo5-NFAT-bla CHO-K1 assay does not have an antagonist control.

CCKAR - NFAT-bla HEK 293T - Agonist Screen
CCKAR-NFAT-bla HEK 293T cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of CCK-8 (control agonist starting concentration, 100 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CCKAR - NFAT-bla HEK 293T - Antagonist Screen, Activated by CCK-8
CCKAR-NFAT-bla HEK 293T cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Lorglumide (control antagonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist CCK-8 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CCKBR - NFAT-bla HEK 293T - Agonist Screen
CCKBR-NFAT-bla HEK 293T cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 156,250 cells/mL. 32 µL of cell suspension (5,000 cells) is added to each well of a 384-well Poly-D-Lysine assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of CCK-8 (control agonist starting concentration, 10 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CCKBR - NFAT-bla HEK 293T - Antagonist Screen, Activated by CCK-8
CCKBR-NFAT-bla HEK 293T cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well Poly-D-Lysine assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist CCK-8 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the CCKBR-NFAT-bla HEK 293T assay does not have an antagonist control.

CCR1 - bla U2OS - Agonist Screen
CCR1-bla U2OS cells are thawed and resuspended in Assay Media (Freestyle media) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Mip1-alpha (control agonist starting concentration, 300 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CCR1 - bla U2OS - Antagonist Screen, Activated by Mip1-alpha
CCR1-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of J113863 (control antagonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Mip1-alpha at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CCR2 - bla U2OS - Agonist Screen
CCR2-bla U2OS cells are thawed and resuspended in Assay Media (Freestyle media) to a concentration of 312,500 cells/mL. 4 µL of a 10X serial dilution of MCP-1 (control agonist starting concentration, 100 nM) or compounds are added to appropriate wells of a 384-well TC-Treated assay plate. 32 µL of cell suspension (10,000 cells) is added to each well. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 16-24 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CCR2 - bla U2OS - Antagonist Screen, Activated by MCP-1
CCR2-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 4 µL of a 10X serial dilution of BMS CCR2 22 (control antagonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of a TC-Treated assay plate. 32 µL of cell suspension is added to the wells and pre-incubated at 37ºC/5% CO2 in a humidified incubator with compounds and control antagonist titration for 30 minutes. 4 µL of 10X control agonist MCP-1 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 16-24 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CCR3 - bla U2OS - Agonist Screen
CCR3-bla U2OS cells are thawed and resuspended in Assay Media (Freestyle media) to a concentration of 312,500 cells/mL. 4 µL of a 10X serial dilution of Eotaxin (control agonist starting concentration, 625 nM) or compounds are added to appropriate wells of a 384-well TC-Treated assay plate. 32 µL of cell suspension (10,000 cells) is added to each well. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 16-24 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CCR3 - bla U2OS - Antagonist Screen, Activated by Eotaxin
CCR3-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 4 µL of 10X compounds or Assay Media are added to appropriate wells of a TC-Treated assay plate. 32 µL of cell suspension is added to the wells and pre-incubated at 37ºC/5% CO2 in a humidified incubator with compounds and control antagonist titration for 30 minutes. 4 µL of 10X control agonist Eotaxin at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 16-24 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the CCR3-bla U2OS assay does not have an antagonist control.

CCR4 - bla U2OS - Agonist Screen
CCR4-bla U2OS cells are thawed and resuspended in Assay Media (Freestyle media) to a concentration of 468,750 cells/mL. 32 µL of cell suspension (15,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of MDC (control agonist starting concentration, 500 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CCR4 - bla U2OS - Antagonist Screen, Activated by MDC
CCR4-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist MDC at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the CCR4-bla U2OS assay does not have an antagonist control.

CCR5 - bla U2OS - Agonist Screen
CCR5-bla U2OS cells are thawed and resuspended in Assay Media (Freestyle media) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Mip1-alpha (control agonist starting concentration, 1,500 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CCR5 - bla U2OS - Antagonist Screen, Activated by Mip1-alpha
CCR5-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Maraviroc (control antagonist starting concentration, 10 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Mip1-alpha at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CCR6 - bla U2OS - Agonist Screen
CCR6-bla U2OS cells are thawed and resuspended in Assay Media (Freestyle media) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Mip3-alpha (control agonist starting concentration, 125 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CCR6 - bla U2OS - Antagonist Screen, Activated by Mip3-alpha
CCR6-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Mip3-alpha at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the CCR6-bla U2OS assay does not have an antagonist control.

CCR7 - bla U2OS - Agonist Screen
CCR7-bla U2OS cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 4 µL of a 10X serial dilution of Mip3-beta (control agonist starting concentration, 400 nM) or compounds are added to appropriate wells of a 384-well TC-Treated assay plate. 32 µL of cell suspension (10,000 cells) is added to each well. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 16-24 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CCR7 - bla U2OS - Antagonist Screen, Activated by Mip3-beta
CCR7-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 4 µL of 10X compounds or Assay Media are added to appropriate wells of a TC-Treated assay plate. 32 µL of cell suspension is added to the wells and pre-incubated at 37ºC/5% CO2 in a humidified incubator with compounds and control antagonist titration for 30 minutes. 4 µL of 10X control agonist Mip3-beta at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 16-24 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the CCR7-bla U2OS assay does not have an antagonist control.

CMKLR1 - bla U2OS - Agonist Screen
CMKLR1-bla U2OS cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Chemerin (control agonist starting concentration, 150 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CMKLR1 - bla U2OS - Antagonist Screen, Activated by Chemerin
CMKLR1-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Chemerin at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the CMKLR1-bla U2OS assay does not have an antagonist control.

CNR1 - Galpha15-NFAT-bla CHO-K1 - Agonist Screen
CNR1-Galpha15-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (Freestyle media) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well Poly-D-Lysine assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of CP-55940 (control agonist starting concentration, 2,500 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CNR1 - Galpha15-NFAT-bla CHO-K1 - Antagonist Screen, Activated by CP-55940
CNR1-Galpha15-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well Poly-D-Lysine assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of AM251 (control antagonist starting concentration, 5,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist CP-55940 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CNR2 - bla U2OS - Agonist Screen
CNR2-bla U2OS cells are thawed and resuspended in Assay Media (Freestyle media) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of CP-55940 (control agonist starting concentration, 2,500 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CNR2 - bla U2OS - Antagonist Screen, Activated by CP-55940
CNR2-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist CP-55940 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the CNR2-bla U2OS assay does not have an antagonist control.

CRHR1 - CRE-bla CHO-K1 - Agonist Screen
CRHR1-CRE-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of CRF (control agonist starting concentration, 1 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 4 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CRHR1 - CRE-bla CHO-K1 - Antagonist Screen, Activated by CRF
CRHR1-CRE-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Astressin (control antagonist starting concentration, 500 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist CRF at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 4 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CRHR2 - CRE-bla CHO-K1 - Agonist Screen
CRHR2-CRE-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of CRF (control agonist starting concentration, 1 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 4 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CRHR2 - CRE-bla CHO-K1 - Antagonist Screen, Activated by CRF
CRHR2-CRE-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Astressin (control antagonist starting concentration, 500 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist CRF at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 4 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CXCR1 - bla U2OS - Agonist Screen
CXCR1-bla U2OS cells are thawed and resuspended in Assay Media (Freestyle media) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of IL-8 (control agonist starting concentration, 580 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CXCR1 - bla U2OS - Antagonist Screen, Activated by IL-8
CXCR1-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist IL-8 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the CXCR1-bla U2OS assay does not have an antagonist control.

CXCR2 - bla U2OS - Agonist Screen
CXCR2-bla U2OS cells are thawed and resuspended in Assay Media (Freestyle media) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of IL-8 (control agonist starting concentration, 290 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CXCR2 - bla U2OS - Antagonist Screen, Activated by IL-8
CXCR2-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist IL-8 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the CXCR2-bla U2OS assay does not have an antagonist control.

CXCR3 - bla U2OS - Agonist Screen
CXCR3-bla U2OS cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 4 µL of a 10X serial dilution of I-TAC (control agonist starting concentration, 500 nM) or compounds are added to appropriate wells of a 384-well TC-Treated assay plate. 32 µL of cell suspension (10,000 cells) is added to each well. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 16-24 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CXCR3 - bla U2OS - Antagonist Screen, Activated by I-TAC
CXCR3-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 4 µL of 10X compounds or Assay Media are added to appropriate wells of a TC-Treated assay plate. 32 µL of cell suspension is added to the wells and pre-incubated at 37ºC/5% CO2 in a humidified incubator with compounds and control antagonist titration for 30 minutes. 4 µL of 10X control agonist I-TAC at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 16-24 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the CXCR3-bla U2OS assay does not have an antagonist control.

CXCR4 - bla U2OS - Agonist Screen
CXCR4-bla U2OS cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of SDF1-alpha (control agonist starting concentration, 2,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CXCR4 - bla U2OS - Antagonist Screen, Activated by SDF1-alpha
CXCR4-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist SDF1-alpha at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the CXCR4-bla U2OS assay does not have an antagonist control.

CXCR6 - bla U2OS - Agonist Screen
CXCR6-bla U2OS cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of CXCL16 (control agonist starting concentration, 200 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CXCR6 - bla U2OS - Antagonist Screen, Activated by CXCL16
CXCR6-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist CXCL16 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the CXCR6-bla U2OS assay does not have an antagonist control.

CXCR7 - bla U2OS - Agonist Screen
CXCR7-bla U2OS cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of SDF1-alpha (control agonist starting concentration, 200 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CXCR7 - bla U2OS - Antagonist Screen, Activated by SDF1-alpha
CXCR7-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist SDF1-alpha at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the CXCR7-bla U2OS assay does not have an antagonist control.

cysLT2 - NFAT-bla CHO-K1 - Agonist Screen
cysLT2-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of LTD4 (control agonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

cysLT2 - NFAT-bla CHO-K1 - Antagonist Screen, Activated by LTD4
cysLT2-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist LTD4 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the cysLT2-NFAT-bla CHO-K1 assay does not have an antagonist control.

D1 - CRE-bla CHO-K1 - Agonist Screen
D1-CRE-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Dihydrexidine (control agonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

D1 - CRE-bla CHO-K1 - Antagonist Screen, Activated by Dihydrexidine
D1-CRE-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of R(+)-SCH-23390 (control antagonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Dihydrexidine at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

D1 - CRE-bla CHO-K1 - Agonist Screen
D1-CRE-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Dopamine (control agonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

D2 - Gqo5-NFAT-bla CHO-K1 - Agonist Screen
D2-Gqo5-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Apomorphine (control agonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

D2 - Gqo5-NFAT-bla CHO-K1 - Antagonist Screen, Activated by Apomorphine
D2-Gqo5-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Perphenazine (control antagonist starting concentration, 100 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Apomorphine at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

EDG1 - bla U2OS - Agonist Screen
EDG1-bla U2OS cells are grown in Growth Media (McCoy's 5A, 10% dialyzed FBS, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep). Cells are dissociated and resuspended in Assay Media (Freestyle media) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of S1P (control agonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

EDG1 - bla U2OS - Antagonist Screen, Activated by S1P
EDG1-bla U2OS cells are grown and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of VPC23019 (control antagonist starting concentration, 2,500 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist S1P at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

EDG2 - bla U2OS - Agonist Screen
EDG2-bla U2OS cells are thawed and resuspended in Assay Media (Freestyle media) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of LPA (18:1) (control agonist starting concentration, 100,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

EDG2 - bla U2OS - Antagonist Screen, Activated by LPA (18:1)
EDG2-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Ki16425 (control antagonist starting concentration, 100,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist LPA (18:1) at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

EDG3 - Galpha15-NFAT-bla HEK 293T - Agonist Screen
EDG3-Galpha15-NFAT-bla HEK 293T cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well Poly-D-Lysine assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of S1P (control agonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

EDG3 - Galpha15-NFAT-bla HEK 293T - Antagonist Screen, Activated by S1P
EDG3-Galpha15-NFAT-bla HEK 293T cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well Poly-D-Lysine assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of CAY10444 (control antagonist starting concentration, 100,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist S1P at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

EDG4 - bla U2OS - Agonist Screen
EDG4-bla U2OS cells are thawed and resuspended in Assay Media (Freestyle media) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 22-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of LPA (18:1) (control agonist starting concentration, 100,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

EDG4 - bla U2OS - Antagonist Screen, Activated by LPA (18:1)
EDG4-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 22-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist LPA (18:1) at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the EDG4-bla U2OS assay does not have an antagonist control.

EDG6 - bla U2OS - Agonist Screen
EDG6-bla U2OS cells are thawed and resuspended in Assay Media (Freestyle media) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well Poly-D-Lysine assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of S1P (control agonist starting concentration, 2,500 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

EDG6 - bla U2OS - Antagonist Screen, Activated by S1P
EDG6-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well Poly-D-Lysine assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist S1P at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the EDG6-bla U2OS assay does not have an antagonist control.

EDG7 - NFAT-bla HEK 293T - Agonist Screen
EDG7-NFAT-bla HEK 293T cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well Poly-D-Lysine assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of LPA (18:1) (control agonist starting concentration, 25,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

EDG7 - NFAT-bla HEK 293T - Antagonist Screen, Activated by LPA (18:1)
EDG7-NFAT-bla HEK 293T cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well Poly-D-Lysine assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of VPC32183 (control antagonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist LPA (18:1) at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

EDG8 - bla U2OS - Agonist Screen
EDG8-bla U2OS cells are grown in Growth Media (McCoy's 5A, 10% dialyzed FBS, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep). Cells are dissociated and resuspended in Assay Media (Freestyle media) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 48 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of S1P (control agonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

EDG8 - bla U2OS - Antagonist Screen, Activated by S1P
EDG8-bla U2OS cells are grown and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 48 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist S1P at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the EDG8-bla U2OS assay does not have an antagonist control.

EDNRA - bla U2OS - Agonist Screen
EDNRA-bla U2OS cells are thawed and resuspended in Assay Media (Freestyle media) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of ET-1 (control agonist starting concentration, 10 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

EDNRA - bla U2OS - Antagonist Screen, Activated by ET-1
EDNRA-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of BQ-123 (control antagonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist ET-1 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

EDNRA - NFAT-bla HEK 293T - Agonist Screen
EDNRA-NFAT-bla HEK 293T cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 156,250 cells/mL. 32 µL of cell suspension (5,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of ET-1 (control agonist starting concentration, 10 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

EDNRA - NFAT-bla HEK 293T - Antagonist Screen, Activated by ET-1
EDNRA-NFAT-bla HEK 293T cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of BQ-123 (control antagonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist ET-1 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

EDNRB - NFAT-bla HEK 293T - Agonist Screen
EDNRB-NFAT-bla HEK 293T cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 156,250 cells/mL. 32 µL of cell suspension (5,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of ET-1 (control agonist starting concentration, 10 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

EDNRB - NFAT-bla HEK 293T - Antagonist Screen, Activated by ET-1
EDNRB-NFAT-bla HEK 293T cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of BQ-788 (control antagonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist ET-1 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

F2RL1 - bla U2OS - Agonist Screen
F2RL1-bla U2OS cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 4 µL of a 10X serial dilution of SLIGRL-NH2 (control agonist starting concentration, 50,000 nM) or compounds are added to appropriate wells of a 384-well TC-Treated assay plate. 32 µL of cell suspension (10,000 cells) is added to each well. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 16-24 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

F2RL1 - bla U2OS - Antagonist Screen, Activated by SLIGRL-NH2
F2RL1-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 4 µL of 10X compounds or Assay Media are added to appropriate wells of a TC-Treated assay plate. 32 µL of cell suspension is added to the wells and pre-incubated at 37ºC/5% CO2 in a humidified incubator with compounds and control antagonist titration for 30 minutes. 4 µL of 10X control agonist SLIGRL-NH2 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 16-24 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the F2RL1-bla U2OS assay does not have an antagonist control.

FPRL1 - Galpha15-NFAT-bla CHO-K1 - Agonist Screen
FPRL1-Galpha15-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% CD-treated FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA,100 U/mL/100 µg/mL Pen/Strep) to a concentration of 156,250 cells/mL. 32 µL of cell suspension (5,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of WKYMVM peptide (control agonist starting concentration, 500 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

FPRL1 - Galpha15-NFAT-bla CHO-K1 - Antagonist Screen, Activated by WKYMVM peptide
FPRL1-Galpha15-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist WKYMVM peptide at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the FPRL1-Galpha15-NFAT-bla CHO-K1 assay does not have an antagonist control.

GALR1 - bla U2OS - Agonist Screen
GALR1-bla U2OS cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Galanin (control agonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

GALR1 - bla U2OS - Antagonist Screen, Activated by Galanin
GALR1-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Galanin at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the GALR1-bla U2OS assay does not have an antagonist control.

GALR2 - bla U2OS - Agonist Screen
GALR2-bla U2OS cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Galanin (1-30) (control agonist starting concentration, 4,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

GALR2 - bla U2OS - Antagonist Screen, Activated by Galanin (1-30)
GALR2-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Galanin (1-30) at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the GALR2-bla U2OS assay does not have an antagonist control.

GCGR - CRE-bla CHO-K1 - Agonist Screen
GCGR-CRE-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% CD-treated FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA,100 U/mL/100 µg/mL Pen/Strep) to a concentration of 468,750 cells/mL. 32 µL of cell suspension (15,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Glucagon (control agonist starting concentration, 8 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

GCGR - CRE-bla CHO-K1 - Antagonist Screen, Activated by Glucagon
GCGR-CRE-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Glucagon at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the GCGR-CRE-bla CHO-K1 assay does not have an antagonist control.

GHSR - TREx-bla U2OS - Antagonist Screen, Constitutively Activated
GHSR-TREx-bla U2OS cells are thawed and resuspended in Assay Media (Freestyle media, 10 ng/ml Doxycycline) to a concentration of 312,500 cells/mL. 4 µL of a 10X serial dilution of Substance P (control antagonist starting concentration, 3,000 nM) or compounds are added to appropriate wells of a TC-Treated assay plate. 32 µL of cell suspension (10,000 cells) is added to the wells. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 16-24 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

GIPR - CRE-bla HEK 293T - Agonist Screen
GIPR-CRE-bla HEK 293T cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Gastric Inhibitory Peptide (control agonist starting concentration, 0.5 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

GIPR - CRE-bla HEK 293T - Antagonist Screen, Activated by Gastric Inhibitory Peptide
GIPR-CRE-bla HEK 293T cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Gastric Inhibitory Peptide at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the GIPR-CRE-bla HEK 293T assay does not have an antagonist control.

GLP1R - CRE-bla CHO-K1 - Agonist Screen
GLP1R-CRE-bla CHO-K1 cells are grown in Growth Media (DMEM, 10% dialyzed FBS, 25 mM HEPES, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep). Cells are dissociated and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of GLP-1 (control agonist starting concentration, 100 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

GLP1R - CRE-bla CHO-K1 - Antagonist Screen, Activated by GLP-1
GLP1R-CRE-bla CHO-K1 cells are grown and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Excendin-3 (control antagonist starting concentration, 30,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist GLP-1 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

GLP2R - bla U2OS - Agonist Screen
GLP2R-bla U2OS cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 4 µL of a 10X serial dilution of GLP-2 (control agonist starting concentration, 2,000 nM) or compounds are added to appropriate wells of a 384-well TC-Treated assay plate. 32 µL of cell suspension (10,000 cells) is added to each well. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 16-24 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

GLP2R - bla U2OS - Antagonist Screen, Activated by GLP-2
GLP2R-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 4 µL of 10X compounds or Assay Media are added to appropriate wells of a TC-Treated assay plate. 32 µL of cell suspension is added to the wells and pre-incubated at 37ºC/5% CO2 in a humidified incubator with compounds and control antagonist titration for 30 minutes. 4 µL of 10X control agonist GLP-2 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 16-24 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the GLP2R-bla U2OS assay does not have an antagonist control.

GnRHR - NFAT-bla CHO-K1 - Agonist Screen
GnRHR-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% CD-treated FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA,100 U/mL/100 µg/mL Pen/Strep) to a concentration of 468,750 cells/mL. 32 µL of cell suspension (15,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of LH-RH (control agonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

GnRHR - NFAT-bla CHO-K1 - Antagonist Screen, Activated by LH-RH
GnRHR-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of LH-RH antagonist (control antagonist starting concentration, 50,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist LH-RH at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

GPR1 - bla U2OS - Agonist Screen
GPR1-bla U2OS cells are thawed and resuspended in Assay Media (Freestyle media) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 48 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Chemerin (control agonist starting concentration, 10 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 16-24 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

GPR1 - bla U2OS - Antagonist Screen, Activated by Chemerin
GPR1-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 48 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Chemerin at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 16-24 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the GPR1-bla U2OS assay does not have an antagonist control.

GPR10 - NFAT-bla CHO-K1 - Agonist Screen
GPR10-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of PrRP20 (control agonist starting concentration, 25 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

GPR10 - NFAT-bla CHO-K1 - Antagonist Screen, Activated by PrRP20
GPR10-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist PrRP20 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the GPR10-NFAT-bla CHO-K1 assay does not have an antagonist control.

GPR109A - bla U2OS - Agonist Screen
GPR109A-bla U2OS cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Nicotinic Acid (control agonist starting concentration, 500,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

GPR109A - bla U2OS - Antagonist Screen, Activated by Nicotinic Acid
GPR109A-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Nicotinic Acid at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the GPR109A-bla U2OS assay does not have an antagonist control.

GPR119 - TREx-CRE-bla CHO-K1 - Agonist Screen
GPR119-TREx-CRE-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep, 0.1 µg/ml Doxycycline) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of AR231453 (control agonist starting concentration, 5,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

GPR119 - TREx-CRE-bla CHO-K1 - Antagonist Screen, Activated by AR231453
GPR119-TREx-CRE-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist AR231453 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the GPR119-TREx-CRE-bla CHO-K1 assay does not have an antagonist control.

GPR120S - bla U2OS - Agonist Screen
GPR120S-bla U2OS cells are thawed and resuspended in Assay Media (Freestyle media) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 48 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of GW9508 (control agonist starting concentration, 30,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

GPR120S - bla U2OS - Antagonist Screen, Activated by GW9508
GPR120S-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 48 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist GW9508 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the GPR120S-bla U2OS assay does not have an antagonist control.

GPR35 - bla U2OS - Agonist Screen
GPR35-bla U2OS cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Zaprinast (control agonist starting concentration, 100,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

GPR35 - bla U2OS - Antagonist Screen, Activated by Zaprinast
GPR35-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Zaprinast at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the GPR35-bla U2OS assay does not have an antagonist control.

GPR44 (CRTH2) - bla U2OS - Agonist Screen
GPR44 (CRTH2)-bla U2OS cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 4 µL of a 10X serial dilution of Indomethicin (control agonist starting concentration, 150,000 nM) or compounds are added to appropriate wells of a 384-well TC-Treated assay plate. 32 µL of cell suspension (10,000 cells) is added to each well. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 16-24 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

GPR44 (CRTH2) - bla U2OS - Antagonist Screen, Activated by Indomethicin
GPR44 (CRTH2)-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 4 µL of a 10X serial dilution of BAY-u3405 (control antagonist starting concentration, 100,000 nM) or compounds are added to appropriate wells of a TC-Treated assay plate. 32 µL of cell suspension is added to the wells and pre-incubated at 37ºC/5% CO2 in a humidified incubator with compounds and control antagonist titration for 30 minutes. 4 µL of 10X control agonist Indomethicin at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 16-24 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

GPR54 - NFAT-bla CHO-K1 - Agonist Screen
GPR54-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Metastin (45-54) (control agonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

GPR54 - NFAT-bla CHO-K1 - Antagonist Screen, Activated by Metastin (45-54)
GPR54-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Metastin (45-54) at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the GPR54-NFAT-bla CHO-K1 assay does not have an antagonist control.

GPR8 - bla U2OS - Agonist Screen
GPR8-bla U2OS cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Neuropeptide B-29 (control agonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

GPR8 - bla U2OS - Antagonist Screen, Activated by Neuropeptide B-29
GPR8-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Neuropeptide B-29 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the GPR8-bla U2OS assay does not have an antagonist control.

GRPR - NFAT-bla CHO-K1 - Agonist Screen
GRPR-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of GRP (control agonist starting concentration, 10 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

GRPR - NFAT-bla CHO-K1 - Antagonist Screen, Activated by GRP
GRPR-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist GRP at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the GRPR-NFAT-bla CHO-K1 assay does not have an antagonist control.

H1 - NFAT-bla HEK 293T - Agonist Screen
H1-NFAT-bla HEK 293T cells are thawed and resuspended in Assay Media (DMEM, 10% CD-treated FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA,100 U/mL/100 µg/mL Pen/Strep) to a concentration of 156,250 cells/mL. 32 µL of cell suspension (5,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Histamine (control agonist starting concentration, 6,400 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

H1 - NFAT-bla HEK 293T - Antagonist Screen, Activated by Histamine
H1-NFAT-bla HEK 293T cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Triprolidine (control antagonist starting concentration, 100 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Histamine at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

H2 - CRE-bla HEK 293T - Agonist Screen
H2-CRE-bla HEK 293T cells are thawed and resuspended in Assay Media (DMEM, 10% CD-treated FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA,100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Histamine (control agonist starting concentration, 400 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

H2 - CRE-bla HEK 293T - Antagonist Screen, Activated by Histamine
H2-CRE-bla HEK 293T cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Tiotidine (control antagonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Histamine at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

H3 - bla U2OS - Agonist Screen
H3-bla U2OS cells are thawed and resuspended in Assay Media (Freestyle media) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Methylhistamine (control agonist starting concentration, 50,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

H3 - bla U2OS - Antagonist Screen, Activated by Methylhistamine
H3-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Thioperamide (control antagonist starting concentration, 70,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Methylhistamine at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

H4 - bla U2OS - Agonist Screen
H4-bla U2OS cells are thawed and resuspended in Assay Media (Freestyle media) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 22-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of 4-Methylhistamine (control agonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

H4 - bla U2OS - Antagonist Screen, Activated by 4-Methylhistamine
H4-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 22-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Thioperamide (control antagonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist 4-Methylhistamine at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

HCRTR1 - NFAT-bla CHO-K1 - Agonist Screen
HCRTR1-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Orexin A (control agonist starting concentration, 100 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

HCRTR1 - NFAT-bla CHO-K1 - Antagonist Screen, Activated by Orexin A
HCRTR1-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of SB 408124 (control antagonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Orexin A at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

HCRTR2 - Galpha15-NFAT-bla CHO-K1 - Agonist Screen
HCRTR2-Galpha15-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Orexin B (control agonist starting concentration, 200 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

HCRTR2 - Galpha15-NFAT-bla CHO-K1 - Antagonist Screen, Activated by Orexin B
HCRTR2-Galpha15-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Orexin B at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the HCRTR2-Galpha15-NFAT-bla CHO-K1 assay does not have an antagonist control.

HTR1A - bla U2OS - Agonist Screen
HTR1A-bla U2OS cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of 5-HT (control agonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

HTR1A - bla U2OS - Antagonist Screen, Activated by 5-HT
HTR1A-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of WAY-100635 (control antagonist starting concentration, 500 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist 5-HT at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

HTR1A - Galpha15-NFAT-bla CHO-K1 - Agonist Screen
HTR1A-Galpha15-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 468,750 cells/mL. 32 µL of cell suspension (15,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of 5-HT (control agonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 4 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

HTR1A - Galpha15-NFAT-bla CHO-K1 - Antagonist Screen, Activated by 5-HT
HTR1A-Galpha15-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Methiothepin (control antagonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist 5-HT at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 4 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

HTR2A - bla U2OS - Agonist Screen
HTR2A-bla U2OS cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of 5-HT (control agonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

HTR2A - bla U2OS - Antagonist Screen, Activated by 5-HT
HTR2A-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Mianserin (control antagonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist 5-HT at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

HTR2B - NFAT-bla CHO-K1 - Agonist Screen
HTR2B-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of 5-HT (control agonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

HTR2B - NFAT-bla CHO-K1 - Antagonist Screen, Activated by 5-HT
HTR2B-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Clozapine (control antagonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist 5-HT at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

HTR7 - CRE-bla CHO-K1 - Agonist Screen
HTR7-CRE-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of 5-HT (control agonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 4 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

HTR7 - CRE-bla CHO-K1 - Antagonist Screen, Activated by 5-HT
HTR7-CRE-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of SB269970 (control antagonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist 5-HT at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 4 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

LTB4R - Galpha15-NFAT-bla CHO-K1 - Agonist Screen
LTB4R-Galpha15-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Leukotriene B4 (control agonist starting concentration, 5,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 4 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

LTB4R - Galpha15-NFAT-bla CHO-K1 - Antagonist Screen, Activated by Leukotriene B4
LTB4R-Galpha15-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Leukotriene B4 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 4 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the LTB4R-Galpha15-NFAT-bla CHO-K1 assay does not have an antagonist control.

M1 - NFAT-bla CHO-K1 - Agonist Screen
M1-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Carbachol (control agonist starting concentration, 100,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

M1 - NFAT-bla CHO-K1 - Antagonist Screen, Activated by Carbachol
M1-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Scopolamine (control antagonist starting concentration, 600 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Carbachol at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

M2 - bla U2OS - Agonist Screen
M2-bla U2OS cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Carbachol (control agonist starting concentration, 1,000,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

M2 - bla U2OS - Antagonist Screen, Activated by Carbachol
M2-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Scopolamine (control antagonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Carbachol at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

M3 - NFAT-bla CHO-K1 - Agonist Screen
M3-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Carbachol (control agonist starting concentration, 100,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

M3 - NFAT-bla CHO-K1 - Antagonist Screen, Activated by Carbachol
M3-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Scopolamine (control antagonist starting concentration, 600 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Carbachol at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

M4 - bla U2OS - Agonist Screen
M4-bla U2OS cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 4 µL of a 10X serial dilution of Carbachol (control agonist starting concentration, 100,000 nM) or compounds are added to appropriate wells of a 384-well TC-Treated assay plate. 32 µL of cell suspension (10,000 cells) is added to each well. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 16-24 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

M4 - bla U2OS - Antagonist Screen, Activated by Carbachol
M4-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 4 µL of a 10X serial dilution of Scopolamine (control antagonist starting concentration, 600 nM) or compounds are added to appropriate wells of a TC-Treated assay plate. 32 µL of cell suspension is added to the wells and pre-incubated at 37ºC/5% CO2 in a humidified incubator with compounds and control antagonist titration for 30 minutes. 4 µL of 10X control agonist Carbachol at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 16-24 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

M4 - Gqo5-NFAT-bla CHO-K1 - Agonist Screen
M4-Gqo5-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well Poly-D-Lysine assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Carbachol (control agonist starting concentration, 100,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

M4 - Gqo5-NFAT-bla CHO-K1 - Antagonist Screen, Activated by Carbachol
M4-Gqo5-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well Poly-D-Lysine assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of DAMP (control antagonist starting concentration, 600 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Carbachol at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

M5 - NFAT-bla CHO-K1 - Agonist Screen
M5-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Carbachol (control agonist starting concentration, 100,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

M5 - NFAT-bla CHO-K1 - Antagonist Screen, Activated by Carbachol
M5-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Scopolamine (control antagonist starting concentration, 600 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Carbachol at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

MC1R - CRE-bla CHO-K1 - Agonist Screen
MC1R-CRE-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of NDP-alpha-MSH (control agonist starting concentration, 10 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

MC1R - CRE-bla CHO-K1 - Antagonist Screen, Activated by NDP-alpha-MSH
MC1R-CRE-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist NDP-alpha-MSH at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the MC1R-CRE-bla CHO-K1 assay does not have an antagonist control.

MC2R - CRE-bla CHO-K1 - Agonist Screen
MC2R-CRE-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of ACTH (1-24) (control agonist starting concentration, 10 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

MC2R - CRE-bla CHO-K1 - Antagonist Screen, Activated by ACTH (1-24)
MC2R-CRE-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist ACTH (1-24) at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the MC2R-CRE-bla CHO-K1 assay does not have an antagonist control.

MC3R - CRE-bla CHO-K1 - Agonist Screen
MC3R-CRE-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of NDP-alpha-MSH (control agonist starting concentration, 10 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

MC3R - CRE-bla CHO-K1 - Antagonist Screen, Activated by NDP-alpha-MSH
MC3R-CRE-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of SHU9119 (control antagonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist NDP-alpha-MSH at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

MC4R - CRE-bla CHO-K1 - Agonist Screen
MC4R-CRE-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 156,250 cells/mL. 32 µL of cell suspension (5,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of alpha-MSH (control agonist starting concentration, 10 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

MC4R - CRE-bla CHO-K1 - Antagonist Screen, Activated by alpha-MSH
MC4R-CRE-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of SHU9119 (control antagonist starting concentration, 10 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist alpha-MSH at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

MC5R - CRE-bla CHO-K1 - Agonist Screen
MC5R-CRE-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of NDP-alpha-MSH (control agonist starting concentration, 1 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

MC5R - CRE-bla CHO-K1 - Antagonist Screen, Activated by NDP-alpha-MSH
MC5R-CRE-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist NDP-alpha-MSH at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the MC5R-CRE-bla CHO-K1 assay does not have an antagonist control.

MCHR1 (GPR24) - Gqi5-NFAT-bla CHO-K1 - Agonist Screen
MCHR1 (GPR24)-Gqi5-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 156,250 cells/mL. 32 µL of cell suspension (5,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of MCH (control agonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

MCHR1 (GPR24) - Gqi5-NFAT-bla CHO-K1 - Antagonist Screen, Activated by MCH
MCHR1 (GPR24)-Gqi5-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of ATC0175 (control antagonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist MCH at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

MCHR2 - bla U2OS - Agonist Screen
MCHR2-bla U2OS cells are thawed and resuspended in Assay Media (Freestyle media) to a concentration of 312,500 cells/mL. 4 µL of a 10X serial dilution of MCH (6-17) (control agonist starting concentration, 30,000 nM) or compounds are added to appropriate wells of a 384-well TC-Treated assay plate. 32 µL of cell suspension (10,000 cells) is added to each well. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 16-24 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

MCHR2 - bla U2OS - Antagonist Screen, Activated by MCH (6-17)
MCHR2-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 4 µL of 10X compounds or Assay Media are added to appropriate wells of a TC-Treated assay plate. 32 µL of cell suspension is added to the wells and pre-incubated at 37ºC/5% CO2 in a humidified incubator with compounds and control antagonist titration for 30 minutes. 4 µL of 10X control agonist MCH (6-17) at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 16-24 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the MCHR2-bla U2OS assay does not have an antagonist control.

MCHR2 - NFAT-bla CHO-K1 - Agonist Screen
MCHR2-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of MCH (6-17) (control agonist starting concentration, 3,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

MCHR2 - NFAT-bla CHO-K1 - Antagonist Screen, Activated by MCH (6-17)
MCHR2-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist MCH (6-17) at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the MCHR2-NFAT-bla CHO-K1 assay does not have an antagonist control.

MTNR1A - bla U2OS - Agonist Screen
MTNR1A-bla U2OS cells are thawed and resuspended in Assay Media (Freestyle media) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Melatonin (control agonist starting concentration, 10 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

MTNR1A - bla U2OS - Antagonist Screen, Activated by Melatonin
MTNR1A-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Luzindole (control antagonist starting concentration, 100,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Melatonin at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

MTNR1B - bla U2OS - Agonist Screen
MTNR1B-bla U2OS cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 4 µL of a 10X serial dilution of Melatonin (control agonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of a 384-well TC-Treated assay plate. 32 µL of cell suspension (10,000 cells) is added to each well. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 16-24 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

MTNR1B - bla U2OS - Antagonist Screen, Activated by Melatonin
MTNR1B-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 4 µL of 10X compounds or Assay Media are added to appropriate wells of a TC-Treated assay plate. 32 µL of cell suspension is added to the wells and pre-incubated at 37ºC/5% CO2 in a humidified incubator with compounds and control antagonist titration for 30 minutes. 4 µL of 10X control agonist Melatonin at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 16-24 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the MTNR1B-bla U2OS assay does not have an antagonist control.

NMUR1 - NFAT-bla CHO-K1 - Agonist Screen
NMUR1-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 78,125 cells/mL. 32 µL of cell suspension (2,500 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of NMU-25 (control agonist starting concentration, 10 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

NMUR1 - NFAT-bla CHO-K1 - Antagonist Screen, Activated by NMU-25
NMUR1-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist NMU-25 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the NMUR1-NFAT-bla CHO-K1 assay does not have an antagonist control.

NPSR1-A - NFAT-bla CHO-K1 - Agonist Screen
NPSR1-A-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Neuropeptide S (control agonist starting concentration, 300 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

NPSR1-A - NFAT-bla CHO-K1 - Antagonist Screen, Activated by Neuropeptide S
NPSR1-A-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Neuropeptide S at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the NPSR1-A-NFAT-bla CHO-K1 assay does not have an antagonist control.

NPSR1-B - NFAT-bla CHO-K1 - Agonist Screen
NPSR1-B-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Neuropeptide S (control agonist starting concentration, 300 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

NPSR1-B - NFAT-bla CHO-K1 - Antagonist Screen, Activated by Neuropeptide S
NPSR1-B-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Neuropeptide S at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the NPSR1-B-NFAT-bla CHO-K1 assay does not have an antagonist control.

NPY1R - bla U2OS - Agonist Screen
NPY1R-bla U2OS cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Neuropeptide Y (control agonist starting concentration, 30 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

NPY1R - bla U2OS - Antagonist Screen, Activated by Neuropeptide Y
NPY1R-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of GR231118 (control antagonist starting concentration, 500 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Neuropeptide Y at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

NPY2R - bla U2OS - Agonist Screen
NPY2R-bla U2OS cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Neuropeptide Y (control agonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

NPY2R - bla U2OS - Antagonist Screen, Activated by Neuropeptide Y
NPY2R-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of BIIE 0246 (control antagonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Neuropeptide Y at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

NTSR1 - NFAT-bla CHO-K1 - Agonist Screen
NTSR1-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Neurotensin (control agonist starting concentration, 100 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

NTSR1 - NFAT-bla CHO-K1 - Antagonist Screen, Activated by Neurotensin
NTSR1-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Neurotensin at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the NTSR1-NFAT-bla CHO-K1 assay does not have an antagonist control.

OPRD1 - bla U2OS - Agonist Screen
OPRD1-bla U2OS cells are grown in Growth Media (McCoy's 5A, 10% dialyzed FBS, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep). Cells are dissociated and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of SNC80 (control agonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

OPRD1 - bla U2OS - Antagonist Screen, Activated by SNC80
OPRD1-bla U2OS cells are grown and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of SDM25N (control antagonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist SNC80 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

OPRK1 - bla U2OS - Agonist Screen
OPRK1-bla U2OS cells are thawed and resuspended in Assay Media (DMEM, 10% CD-treated FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA,100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of U50488 (control agonist starting concentration, 150 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

OPRK1 - bla U2OS - Antagonist Screen, Activated by U50488
OPRK1-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of nor-BNI (control antagonist starting concentration, 100 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist U50488 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

OPRK1 - Gqo5-NFAT-bla CHO-K1 - Agonist Screen
OPRK1-Gqo5-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of U50488 (control agonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

OPRK1 - Gqo5-NFAT-bla CHO-K1 - Antagonist Screen, Activated by U50488
OPRK1-Gqo5-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of nor-BNI (control antagonist starting concentration, 100 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist U50488 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

OPRL1 - bla U2OS - Agonist Screen
OPRL1-bla U2OS cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Nociceptin (control agonist starting concentration, 100 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

OPRL1 - bla U2OS - Antagonist Screen, Activated by Nociceptin
OPRL1-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of UFP-101 (control antagonist starting concentration, 25,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Nociceptin at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

OPRL1 - Gqi5-NFAT-bla Freestyle 293F - Agonist Screen
OPRL1-Gqi5-NFAT-bla Freestyle 293F cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 234,375 cells/mL. 32 µL of cell suspension (7,500 cells) is added to each well of a 384-well Poly-D-Lysine assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Nociceptin (control agonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

OPRL1 - Gqi5-NFAT-bla Freestyle 293F - Antagonist Screen, Activated by Nociceptin
OPRL1-Gqi5-NFAT-bla Freestyle 293F cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well Poly-D-Lysine assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of UFP-101 (control antagonist starting concentration, 25,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Nociceptin at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

OPRM1 - bla U2OS - Agonist Screen
OPRM1-bla U2OS cells are thawed and resuspended in Assay Media (DMEM, 10% CD-treated FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA,100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 4 µL of a 10X serial dilution of DAMGO (control agonist starting concentration, 3,000 nM) or compounds are added to appropriate wells of a 384-well TC-Treated assay plate. 32 µL of cell suspension (10,000 cells) is added to each well. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 16-24 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

OPRM1 - bla U2OS - Antagonist Screen, Activated by DAMGO
OPRM1-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 4 µL of a 10X serial dilution of beta-funaltrexamine (control antagonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of a TC-Treated assay plate. 32 µL of cell suspension is added to the wells and pre-incubated at 37ºC/5% CO2 in a humidified incubator with compounds and control antagonist titration for 30 minutes. 4 µL of 10X control agonist DAMGO at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 16-24 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

OXTR - Gqo5-NFAT-bla CHO-K1 - Agonist Screen
OXTR-Gqo5-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Oxytocin (control agonist starting concentration, 2,500 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

OXTR - Gqo5-NFAT-bla CHO-K1 - Antagonist Screen, Activated by Oxytocin
OXTR-Gqo5-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Atosiban (control antagonist starting concentration, 50,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Oxytocin at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

P2RY11 - NFAT-bla CHO-K1 - Agonist Screen
P2RY11-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% CD-treated FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA,100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of ATP (control agonist starting concentration, 20,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

P2RY11 - NFAT-bla CHO-K1 - Antagonist Screen, Activated by ATP
P2RY11-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Suramin (control antagonist starting concentration, 1,000,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist ATP at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

P2RY2 - NFAT-bla CHO-K1 - Agonist Screen
P2RY2-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% CD-treated FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA,100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of ATP (control agonist starting concentration, 30,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

P2RY2 - NFAT-bla CHO-K1 - Antagonist Screen, Activated by ATP
P2RY2-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Suramin (control antagonist starting concentration, 1,000,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist ATP at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

P2RY6 - NFAT-bla CHO-K1 - Agonist Screen
P2RY6-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% CD-treated FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA,100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of UDP (control agonist starting concentration, 3,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

P2RY6 - NFAT-bla CHO-K1 - Antagonist Screen, Activated by UDP
P2RY6-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Suramin (control antagonist starting concentration, 1,000,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist UDP at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

PAC1 - CRE-bla CHO-K1 - Agonist Screen
PAC1-CRE-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of PACAP (control agonist starting concentration, 1 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

PAC1 - CRE-bla CHO-K1 - Antagonist Screen, Activated by PACAP
PAC1-CRE-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist PACAP at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the PAC1-CRE-bla CHO-K1 assay does not have an antagonist control.

PTAFR - bla U2OS - Agonist Screen
PTAFR-bla U2OS cells are thawed and resuspended in Assay Media (Freestyle media) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of PAF (control agonist starting concentration, 500 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

PTAFR - bla U2OS - Antagonist Screen, Activated by PAF
PTAFR-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of ABT-491 (control antagonist starting concentration, 5 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist PAF at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

PTGDR - CRE-bla CHO-K1 - Agonist Screen
PTGDR-CRE-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of PGD2 (control agonist starting concentration, 40 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

PTGDR - CRE-bla CHO-K1 - Antagonist Screen, Activated by PGD2
PTGDR-CRE-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of AH6809 (control antagonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist PGD2 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

PTGER1 - NFAT-bla CHO-K1 - Agonist Screen
PTGER1-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% CD-treated FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA,100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of PGE2 (control agonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

PTGER1 - NFAT-bla CHO-K1 - Antagonist Screen, Activated by PGE2
PTGER1-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of AH6809 (control antagonist starting concentration, 50,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist PGE2 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

PTGER2 - CRE-bla CHO-K1 - Agonist Screen
PTGER2-CRE-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of PGE2 (control agonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

PTGER2 - CRE-bla CHO-K1 - Antagonist Screen, Activated by PGE2
PTGER2-CRE-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of AH6809 (control antagonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist PGE2 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

PTGIR - CRE-bla CHO-K1 - Agonist Screen
PTGIR-CRE-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% CD-treated FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA,100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Iloprost (control agonist starting concentration, 200 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

PTGIR - CRE-bla CHO-K1 - Antagonist Screen, Activated by Iloprost
PTGIR-CRE-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of CAY10441 (control antagonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Iloprost at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

RLN3R1 - bla U2OS - Agonist Screen
RLN3R1-bla U2OS cells are thawed and resuspended in Assay Media (Freestyle media) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Relaxin-3 (control agonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

RLN3R1 - bla U2OS - Antagonist Screen, Activated by Relaxin-3
RLN3R1-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Relaxin-3 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the RLN3R1-bla U2OS assay does not have an antagonist control.

SCTR - CRE-bla CHO-K1 - Agonist Screen
SCTR-CRE-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Secretin (control agonist starting concentration, 10 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

SCTR - CRE-bla CHO-K1 - Antagonist Screen, Activated by Secretin
SCTR-CRE-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Secretin at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the SCTR-CRE-bla CHO-K1 assay does not have an antagonist control.

SSTR1 - bla U2OS - Agonist Screen
SSTR1-bla U2OS cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of SST14 (control agonist starting concentration, 610 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

SSTR1 - bla U2OS - Antagonist Screen, Activated by SST14
SSTR1-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist SST14 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the SSTR1-bla U2OS assay does not have an antagonist control.

SSTR2 - bla U2OS - Agonist Screen
SSTR2-bla U2OS cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of SST14 (control agonist starting concentration, 610 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

SSTR2 - bla U2OS - Antagonist Screen, Activated by SST14
SSTR2-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of CYN154806 (control antagonist starting concentration, 5,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist SST14 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

SSTR5 - bla U2OS - Agonist Screen
SSTR5-bla U2OS cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of SST14 (control agonist starting concentration, 610 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

SSTR5 - bla U2OS - Antagonist Screen, Activated by SST14
SSTR5-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist SST14 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the SSTR5-bla U2OS assay does not have an antagonist control.

TACR1 - bla U2OS - Agonist Screen
TACR1-bla U2OS cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Substance P (control agonist starting concentration, 300 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

TACR1 - bla U2OS - Antagonist Screen, Activated by Substance P
TACR1-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist Substance P at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the TACR1-bla U2OS assay does not have an antagonist control.

TACR2 - NFAT-bla CHO-K1 - Agonist Screen
TACR2-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of (Nle10)-Neurokinin A (4-10) (control agonist starting concentration, 300 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

TACR2 - NFAT-bla CHO-K1 - Antagonist Screen, Activated by (Nle10)-Neurokinin A (4-10)
TACR2-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of MEN-10376 (control antagonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist (Nle10)-Neurokinin A (4-10) at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

TACR3 - bla U2OS - Agonist Screen
TACR3-bla U2OS cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of SSP Frag 6-11 (control agonist starting concentration, 312.5 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

TACR3 - bla U2OS - Antagonist Screen, Activated by SSP Frag 6-11
TACR3-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist SSP Frag 6-11 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the TACR3-bla U2OS assay does not have an antagonist control.

TBXA2R - bla U2OS - Agonist Screen
TBXA2R-bla U2OS cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of U46619 (control agonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

TBXA2R - bla U2OS - Antagonist Screen, Activated by U46619
TBXA2R-bla U2OS cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of L655240 (control antagonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist U46619 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

VPAC1 - CRE-bla CHO-K1 - Agonist Screen
VPAC1-CRE-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of VIP (control agonist starting concentration, 10 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

VPAC1 - CRE-bla CHO-K1 - Antagonist Screen, Activated by VIP
VPAC1-CRE-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of VIP1 Antagonist (control antagonist starting concentration, 1,000 nM) or compounds are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist VIP at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

VPAC2 - CRE-bla CHO-K1 - Agonist Screen
VPAC2-CRE-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 1% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 32 µL of cell suspension (10,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of VIP (control agonist starting concentration, 10 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

VPAC2 - CRE-bla CHO-K1 - Antagonist Screen, Activated by VIP
VPAC2-CRE-bla CHO-K1 cells are thawed and prepared as described above for the Agonist Screen. 32 µL of cell suspension is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of 10X compounds or Assay Media are added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO2 in a humidified incubator with cells for 30 minutes. 4 µL of 10X control agonist VIP at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. At this time, the VPAC2-CRE-bla CHO-K1 assay does not have an antagonist control.

Parental Cell Line-Specific Assay Conditions

CRE-bla CHO-K1 - Agonist Screen
CRE-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 625,000 cells/mL. 32 µL of cell suspension (20,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Forskolin (control agonist starting concentration, 10,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CRE-bla Freestyle 293F - Agonist Screen
CRE-bla Freestyle 293F cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 625,000 cells/mL. 32 µL of cell suspension (20,000 cells) is added to each well of a 384-well Poly-D-Lysine assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Forskolin (control agonist starting concentration, 40,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

CRE-bla HEK 293T - Agonist Screen
CRE-bla HEK 293T cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 625,000 cells/mL. 32 µL of cell suspension (20,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Forskolin (control agonist starting concentration, 25,000 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

Galpha15-NFAT-bla CHO-K1 - Agonist Screen
Galpha15-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 156,250 cells/mL. 32 µL of cell suspension (5,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Thapsigargin (control agonist starting concentration, 80 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

Galpha15-NFAT-bla HEK 293T - Agonist Screen
Galpha15-NFAT-bla HEK 293T cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 156,250 cells/mL. 32 µL of cell suspension (5,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Thapsigargin + 5 nM PMA (control agonist starting concentration, 100 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

Gqi5-NFAT-bla Freestyle 293F - Agonist Screen
Gqi5-NFAT-bla Freestyle 293F cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 625,000 cells/mL. 32 µL of cell suspension (20,000 cells) is added to each well of a 384-well Poly-D-Lysine assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of PMA (control agonist starting concentration, 100 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

Gqo5-NFAT-bla CHO-K1 - Agonist Screen
Gqo5-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 156,250 cells/mL. 32 µL of cell suspension (5,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Thapsigargin (control agonist starting concentration, 80 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

NFAT-bla CHO-K1 - Agonist Screen
NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 156,250 cells/mL. 32 µL of cell suspension (5,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Thapsigargin (control agonist starting concentration, 80 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

NFAT-bla HEK 293T - Agonist Screen
NFAT-bla HEK 293T cells are thawed and resuspended in Assay Media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 156,250 cells/mL. 32 µL of cell suspension (5,000 cells) is added to each well of a 384-well TC-Treated assay plate. Cells in Assay Media are incubated for 16-24 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Thapsigargin + 5 nM PMA (control agonist starting concentration, 100 nM) or compounds are added to appropriate wells of the plate. 4 µL of Assay Media is added to all wells to bring the final assay volume to 40 µL. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate + Solution D Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.