ThermoFisher SCIENTIFIC

Screening Protocol and Assay Conditions

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Overview and Assay Theory

The SelectScreen Cell-based Pathway Profiling Service utilizes Life Technologies' growing library of GeneBLAzer™ cell signaling pathway specific CellSensor™ cell lines that are cryo-preserved and our robust GeneBLAzer Beta-lactamase (*bla*) Reporter Technology. The Service provides you with a reliable, rapid, and sensitive method of analyzing the intracellular status of a wide range of disease-relevant signal transduction pathways upon exposure to drug candidates or other stimuli. When the pathway is activated or inhibited, Beta-lactamase reporter activity is modulated and can be measured quantitatively and selectively with the LiveBLAzer™-FRET B/G Loading Substrate.

All cell signaling pathway specific CellSensor cell lines used in the SelectScreen Cell-based Pathway Profiling Service provide superior response profiles as a result of response element optimization and use of optimal clones. All CellSensor cell lines in the portfolio were researched and developed by Life Technologies; therefore, we ensure the consistency, reliability and performance of each cell line. The CellSensor cell lines:

- Provide ready-to-screen, ratiometric assays for disease relevant targets
- Are functionally validated to ensure high-quality results each and every time

The CellSensor cell lines in the Service are tested and documented to show a high level of performance. These assays are shown to meet the following specifications:

- Z′-factor of 0.5 or greater for activator assays and Z′-factor of 0.4 or greater for inhibitor assays
- Appropriate EC_{50}/IC_{50} responses to known activators and inhibitors

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

How GeneBLAzer Technology works

GeneBLAzer Technology uses 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.

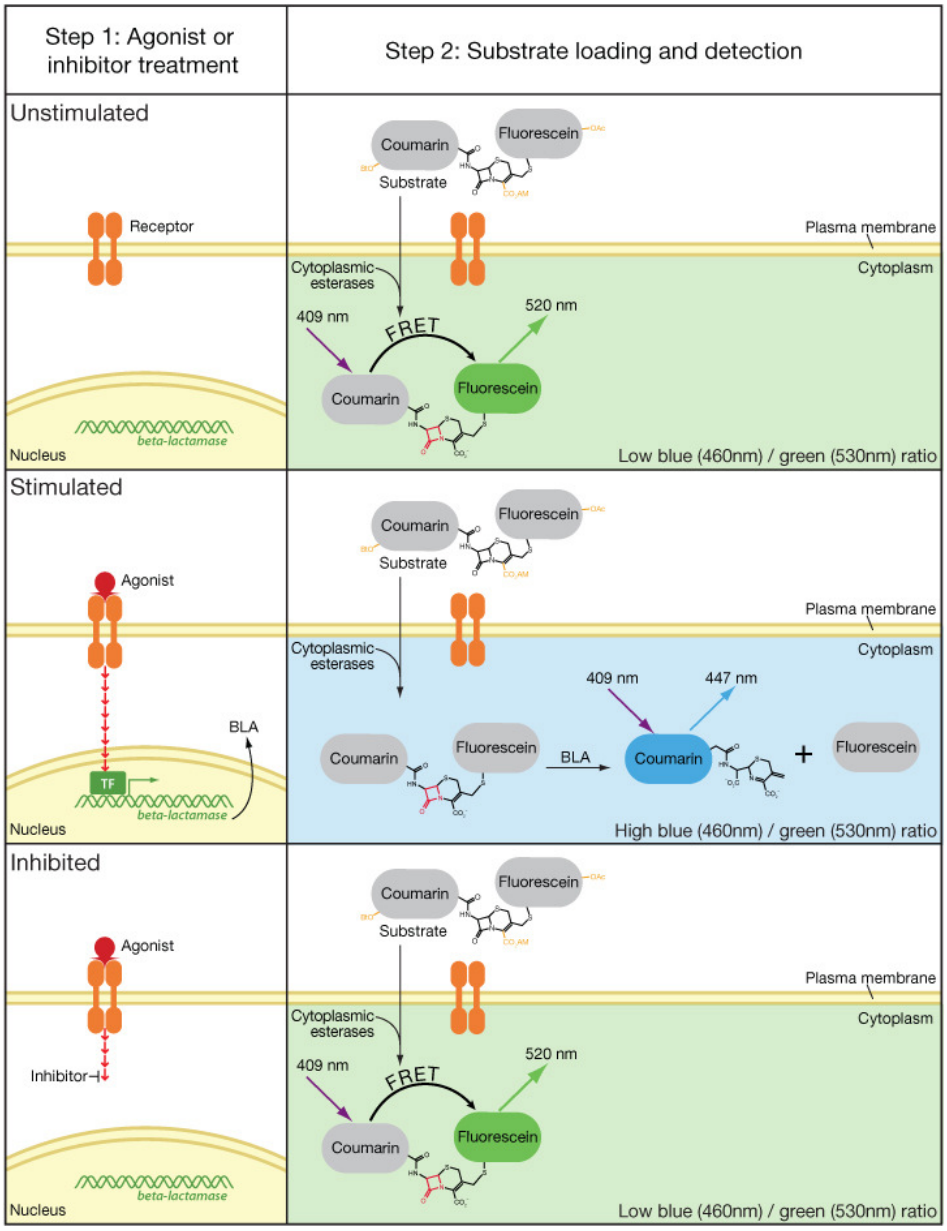

Figure 1. Fluorescent detection of cells using the GeneBLAzer technology. LiveBLAzer™ FRET B/G substrate enters the cell where cleavage by endogenous cytoplasmic esterases converts the LiveBLAzer™ FRET B/G substrate to its negative form trapping it within the cytosol of the cell. In the absence of beta-lactamase activity excitation of the coumarin moiety results in FRET to the fluorescein resulting in a green fluorescent signal. In the presence of beta-lactamase the LiveBLAzer™ FRET B/G substrate is cleaved separating the two dyes disrupting FRET resulting in a blue fluorescent signal.

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.

Activator 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.

Inhibitor Assay Protocol

An activator 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 inhibitor 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 activator, as determined in an Activator 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 Medium 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 CellSensor cell-line:

Full Activator control

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

No Activator control

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

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

Known Activator (activator mode) or Inhibitor (inhibitor mode) Titration

A known activator or inhibitor titration is run on every assay 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)	$\dfrac{\textbf{Emission Ratio}_{\text{Compound}}}{\textbf{Emission Ratio}_{\text{No Act. Ctrl}}}$
% Activation – Activator Assays	$\left\{ \dfrac{\textbf{Response Ratio}_{\text{Compound}} - \textbf{Response Ratio}_{\text{No Act. Ctrl}}}{\textbf{Response Ratio}_{\text{Full Act. Ctrl}} - \textbf{Response Ratio}_{\text{No Act. Ctrl}}} \right\} * 100$
% Inhibition – Inhibitor Assays	$\left\{ 1 - \dfrac{\textbf{Response Ratio}_{\text{Compound}} - \textbf{Response Ratio}_{\text{No Act. Ctrl}}}{\textbf{Response Ratio}_{\text{EC80 Ctrl}} - \textbf{Response Ratio}_{\text{No Act. Ctrl}}} \right\} * 100$
Z′ - Activator Assays (using Emission Ratio values)	$1 - \dfrac{\textbf{3*Std Dev}_{\text{Full Act. Ctrl}} + \textbf{3*Std Dev}_{\text{No Act. Ctrl}}}{\textbf{Mean}_{\text{Full Act. Ctrl}} - \textbf{Mean}_{\text{No Act. Ctrl}}}$
Z′ - Inhibitor Assays (using Emission Ratio values)	$1 - \dfrac{\textbf{3*Std Dev}_{\text{EC80 Ctrl}} + \textbf{3*Std Dev}_{\text{No Act. Ctrl}}}{\textbf{Mean}_{\text{EC80 Ctrl}} - \textbf{Mean}_{\text{No Act. Ctrl}}}$

Graphing Software

SelectScreen Cell-Based Pathway 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.

Pathway Profiling Cell Lines Available for Screening

Assay	Cell Line	Tech-nology	Activator	EC50 (nM)	Inhibitor	IC50 (nM)	Act. Mode	Inh. Mode
B cell receptor	NFAT-bla RA1	BLA	Goat anti-Human IgM	1.30	Syk Inhibitor II	208	Yes	Yes
cAMP/PKA	CRE-bla HEK 293T	BLA	Forskolin	273	H-89	5,250	Yes	Yes
cAMP/PKA	CRE-bla Jurkat	BLA	Forskolin	10,000	H-89	6,108	Yes	Yes
DNA damage/p53 Response	p53RE-bla HCT-116	BLA	Mitomycin C	730			Yes	
ER Stress	ESRE-bla HeLa	BLA	Tunicamycin	176	None	---	Yes	Yes
Glucocorticoid Receptor	MMTV-bla HeLa	BLA	Dexamethasone	6.65	RU-486	1.85	Yes	Yes
Heat Shock	HSE-bla HeLa	BLA	17-AAG	27.0	Quercetin	6,390	Yes	Yes
Hypoxia	HRE-bla ME-180	BLA	CoCl2	24,200	Chetomin	10.6	Yes	Yes
Interleukin 4/STAT6	STAT6-bla RA1	BLA	IL-4	0.004	JAK Inhibitor I	12.1	Yes	Yes
JAK/STAT	irf1-bla HEL	BLA	None		JAK Inhibitor I	449		Yes
JAK/STAT	ISRE-bla HEK 293T	BLA	IFN-alpha	0.006	JAK Inhibitor I	25.3	Yes	Yes
JAK/STAT	ISRE-bla Jurkat	BLA	IFN-alpha	0.006	JAK Inhibitor I	19.9	Yes	Yes
JAK/STAT	SIE-bla HEK 293T	BLA	IFN-gamma	0.006	JAK Inhibitor I	62.4	Yes	Yes
JAK/STAT	SIE-bla HEK 293T	BLA	IL-6	0.107	JAK Inhibitor I	33.1	Yes	Yes
JAK/STAT	SIE-bla ME-180	BLA	IFN-gamma	0.005	JAK Inhibitor I	95.0	Yes	Yes
JAK/STAT	SIE-bla ME-180	BLA	IL-6	0.085	JAK Inhibitor I	15.6	Yes	Yes
JAK2/STAT5	irf1-bla TF1	BLA	EPO	0.053	JAK Inhibitor I	8.94	Yes	Yes
JAK2/STAT5	irf1-bla TF1	BLA	GM-CSF	0.012	JAK Inhibitor I	26.2	Yes	Yes
MAPK	c-fos-bla HEK 293T	BLA	PMA	0.264	U0126	682	Yes	Yes
MAPK	c-fos-bla ME-180	BLA	EGF	0.090	PD153035	72.1	Yes	Yes
MAPK/EGFR/Ras/Raf	AP1-bla ME-180	BLA	EGF	0.051	PD153035	48.7	Yes	Yes
MAPK/MEK/B-raf	AP1-bla A375	BLA	None		Raf1 Kinase Inhibitor	3,690		Yes
NFKB	NFKB-bla Jurkat	BLA	TNF-alpha	0.3993	Withaferin A	283	Yes	Yes
NFkB	NFkB-bla ME-180	BLA	IL-1 beta	0.015	Withaferin A	275	Yes	Yes
NFkB	NFkB-bla ME-180	BLA	TNF-alpha	0.011	Withaferin A	514	Yes	Yes
NFkB	NFkB-bla THP-1	BLA	TNF-alpha	0.007	Withaferin A	649	Yes	Yes
Oxidative Stress	ARE-bla HepG2	BLA	tBHQ	9,060	Ro-31-8220	2,590	Yes	Yes
PI3K/AKT/FOXO3	TREx FOXO3-DBE-bla HeLa	BLA	Insulin	2.31	Triciribine	85.1	Yes	Yes
PKC/Ca2+	NFAT-bla Jurkat	BLA	Thapsigargin	4.63	Ro-31-8220	655	Yes	Yes
PKC/Ca2+	TrkA-NFAT-bla CHO-K1	BLA	NGF 2.5S	0.117	K252a	7.03	Yes	Yes
PKC/Ca2+	TrkB-NFAT-bla CHO-K1	BLA	BDNF	0.127	K252a	6.90	Yes	Yes
PKC/Ca2+	TrkC-NFAT-bla CHO-K1	BLA	Neurotrophin-3	0.151	K252a	4.22	Yes	Yes
T cell receptor	NFAT-bla Jurkat	BLA	anti-CD3:CD28 *	0.230	Ro-31-8220	621	Yes	Yes
TGF-beta	SBE-bla HEK 293T	BLA	TGF-beta 1	0.008	TGF-beta R1	32.8	Yes	Yes

TNF-alpha/JNK	AP1-bla ME-180	BLA	TNF-alpha	0.044	JNK Inhibitor II	5,660	Yes	Yes
Toll-like Receptor (TLR4)	NFkB-bla THP-1	BLA	LPS	0.001	Withaferin A	246	Yes	Yes
Wnt/Beta-Catenin	LEF-TCF-bla HCT116	BLA	Mouse Rec. Wnt-3A	0.042	ICG-001	869	Yes	Yes
Wnt/Beta-Catenin (APC-/-)	LEF-TCF-bla SW480	BLA	None		ICG-001	475		Yes

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

Cell Line-Specific Assay Conditions

B cell receptor - NFAT-bla RA1 - Activator Screen
NFAT-bla RA1 cells are thawed and resuspended in Assay Media (OPTI-MEM, 0.5% dialyzed FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 1,562,500 cells/mL. 4 µL of a 10X serial dilution of Goat anti-Human IgM (control activator starting concentration, 11 nM) or compounds are added to appropriate wells of a 384-well Poly-D-Lysine assay plate. 32 µL of cell suspension (50,000 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 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

B cell receptor - NFAT-bla RA1 - Inhibitor Screen, Activated by Goat anti-Human IgM
NFAT-bla RA1 cells are thawed and prepared as described above for the Activator Screen. 4 µL of a 10X serial dilution of Syk Inhibitor II (control inhibitor starting concentration, 10,000 nM) or compounds are added to appropriate wells of a Poly-D-Lysine 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 inhibitor titration for 30 minutes. 4 µL of 10X control activator Goat anti-Human IgM at the pre-determined EC80 concentration is added to wells containing the control inhibitor or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

cAMP/PKA - CRE-bla HEK 293T - Activator Screen
CRE-bla HEK 293T cells are thawed and resuspended in Assay Media (OPTI-MEM, 0.5% dialyzed FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 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 Forskolin (control activator 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

cAMP/PKA - CRE-bla HEK 293T - Inhibitor Screen, Activated by Forskolin
CRE-bla HEK 293T cells are thawed and prepared as described above for the Activator 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 H-89 (control inhibitor 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 activator Forskolin at the pre-determined EC80 concentration is added to wells containing the control inhibitor or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

cAMP/PKA - CRE-bla Jurkat - Activator Screen
CRE-bla Jurkat cells are thawed and resuspended in Assay Media (OPTI-MEM, 0.5% dialyzed FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 625,000 cells/mL. 4 µL of a 10X serial dilution of Forskolin (control activator 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 (20,000 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 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

cAMP/PKA - CRE-bla Jurkat - Inhibitor Screen, Activated by Forskolin
CRE-bla Jurkat cells are thawed and prepared as described above for the Activator Screen. 4 µL of a 10X serial dilution of H-89 (control inhibitor 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 inhibitor titration for 30 minutes. 4 µL of 10X control activator Forskolin at the pre-determined EC80 concentration is added to wells containing the control inhibitor or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

DNA damage/p53 Response - p53RE-bla HCT-116 - Activator Screen
p53RE-bla HCT-116 cells are thawed and resuspended in Assay Media (OPTI-MEM, 0.5% dialyzed FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 781,250 cells/mL. 32 µL of cell suspension (25,000 cells) is added to each well of a 384-well Poly-D-Lysine assay plate. Cells in Assay Media are incubated for 6 hours in the plate at 37ºC/5% CO2 in a humidified incubator. 4 µL of a 10X serial dilution of Mitomycin C (control activator 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 16-24 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

ER Stress - ESRE-bla HeLa - Activator Screen
ESRE-bla HeLa cells are thawed and resuspended in Assay Media (OPTI-MEM, 0.5% dialyzed FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 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 Tunicamycin (control activator 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

ER Stress - ESRE-bla HeLa - Inhibitor Screen, Activated by Tunicamycin
ESRE-bla HeLa cells are thawed and prepared as described above for the Activator 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 None (control inhibitor starting concentration, 0 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 activator Tunicamycin at the pre-determined EC80 concentration is added to wells containing the control inhibitor or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

Glucocorticoid Receptor - MMTV-bla HeLa - Activator Screen
MMTV-bla HeLa cells are thawed and resuspended in Assay Media (OPTI-MEM, 0.5% dialyzed FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 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 Dexamethasone (control activator 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.

Glucocorticoid Receptor - MMTV-bla HeLa - Inhibitor Screen, Activated by Dexamethasone
MMTV-bla HeLa cells are thawed and prepared as described above for the Activator 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 RU-486 (control inhibitor 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 activator Dexamethasone at the pre-determined EC80 concentration is added to wells containing the control inhibitor 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.

Heat Shock - HSE-bla HeLa - Activator Screen
HSE-bla HeLa cells are thawed and resuspended in Assay Media (DMEM, 0.1% dialyzed FBS, 0.1 mM NEAA, 25 mM HEPES pH 7.3, 100 U/mL/100 ug/mL Pen/Strep) to a concentration of 250,000 cells/mL. 32 µL of cell suspension (8,000 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 17-AAG (control activator 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

Heat Shock - HSE-bla HeLa - Inhibitor Screen, Activated by 17-AAG
HSE-bla HeLa cells are thawed and prepared as described above for the Activator 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 Quercetin (control inhibitor 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 activator 17-AAG at the pre-determined EC80 concentration is added to wells containing the control inhibitor or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

Hypoxia - HRE-bla ME-180 - Activator Screen
HRE-bla ME-180 cells are thawed and resuspended in Assay Media (OPTI-MEM, 0.5% dialyzed FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 187,500 cells/mL. 4 µL of a 10X serial dilution of CoCl2 (control activator starting concentration, 150,000 nM) or compounds are added to appropriate wells of a 384-well Poly-D-Lysine assay plate. 32 µL of cell suspension (6,000 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 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

Hypoxia - HRE-bla ME-180 - Inhibitor Screen, Activated by CoCl2
HRE-bla ME-180 cells are thawed and prepared as described above for the Activator Screen. 4 µL of a 10X serial dilution of Chetomin (control inhibitor starting concentration, 100 nM) or compounds are added to appropriate wells of a Poly-D-Lysine 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 inhibitor titration for 30 minutes. 4 µL of 10X control activator CoCl2 at the pre-determined EC80 concentration is added to wells containing the control inhibitor or compounds. The plate is incubated for 16 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

Interleukin 4/STAT6 - STAT6-bla RA1 - Activator Screen
STAT6-bla RA1 cells are thawed and resuspended in Assay Media (OPTI-MEM, 0.5% dialyzed FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 100 U/mL/100 µg/mL Pen/Strep, 550 ng/mL CD40L) to a concentration of 781,250 cells/mL. 32 µL of cell suspension (25,000 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 IL-4 (control activator starting concentration, 6.7 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

Interleukin 4/STAT6 - STAT6-bla RA1 - Inhibitor Screen, Activated by IL-4
STAT6-bla RA1 cells are thawed and prepared as described above for the Activator 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 JAK Inhibitor I (control inhibitor 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 activator IL-4 at the pre-determined EC80 concentration is added to wells containing the control inhibitor or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

JAK/STAT - irf1-bla HEL - Inhibitor Screen, Constitutively Activated
irf1-bla HEL cells are thawed and resuspended in Assay Media (RPMI, 10% dialyzed FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 781,250 cells/mL. 4 µL of a 10X serial dilution of JAK Inhibitor I (control inhibitor starting concentration, 10,000 nM) or compounds are added to appropriate wells of a TC-Treated assay plate. 32 µL of cell suspension (25,000 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 Loading Solution is added to each well and the plate is incubated for 4 hours at room temperature. The plate is read on a fluorescence plate reader.

JAK/STAT - ISRE-bla HEK 293T - Activator Screen
ISRE-bla HEK 293T cells are thawed and resuspended in Assay Media (OPTI-MEM, 0.5% dialyzed FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 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 IFN-alpha (control activator 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

JAK/STAT - ISRE-bla HEK 293T - Inhibitor Screen, Activated by IFN-alpha
ISRE-bla HEK 293T cells are thawed and prepared as described above for the Activator 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 JAK Inhibitor I (control inhibitor 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 activator IFN-alpha at the pre-determined EC80 concentration is added to wells containing the control inhibitor or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

JAK/STAT - ISRE-bla Jurkat - Activator Screen
ISRE-bla Jurkat cells are thawed and resuspended in Assay Media (OPTI-MEM, 0.5% dialyzed FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 625,000 cells/mL. 4 µL of a 10X serial dilution of IFN-alpha (control activator starting concentration, 0.5 nM) or compounds are added to appropriate wells of a 384-well TC-Treated assay plate. 32 µL of cell suspension (20,000 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 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 3 hours at room temperature. The plate is read on a fluorescence plate reader.

JAK/STAT - ISRE-bla Jurkat - Inhibitor Screen, Activated by IFN-alpha
ISRE-bla Jurkat cells are thawed and prepared as described above for the Activator Screen. 4 µL of a 10X serial dilution of JAK Inhibitor I (control inhibitor 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 inhibitor titration for 30 minutes. 4 µL of 10X control activator IFN-alpha at the pre-determined EC80 concentration is added to wells containing the control inhibitor or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 3 hours at room temperature. The plate is read on a fluorescence plate reader.

JAK/STAT - SIE-bla HEK 293T - Activator Screen
SIE-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 IFN-gamma (control activator 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

JAK/STAT - SIE-bla HEK 293T - Inhibitor Screen, Activated by IFN-gamma
SIE-bla HEK 293T cells are thawed and prepared as described above for the Activator 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 JAK Inhibitor I (control inhibitor 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 activator IFN-gamma at the pre-determined EC80 concentration is added to wells containing the control inhibitor or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

JAK/STAT - SIE-bla HEK 293T - Activator Screen
SIE-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 IL-6 (control activator starting concentration, 20 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

JAK/STAT - SIE-bla HEK 293T - Inhibitor Screen, Activated by IL-6
SIE-bla HEK 293T cells are thawed and prepared as described above for the Activator 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 JAK Inhibitor I (control inhibitor 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 activator IL-6 at the pre-determined EC80 concentration is added to wells containing the control inhibitor or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

JAK/STAT - SIE-bla ME-180 - Activator Screen
SIE-bla ME-180 cells are thawed and resuspended in Assay Media (OPTI-MEM, 0.5% dialyzed FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 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 IFN-gamma (control activator 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

JAK/STAT - SIE-bla ME-180 - Inhibitor Screen, Activated by IFN-gamma
SIE-bla ME-180 cells are thawed and prepared as described above for the Activator 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 JAK Inhibitor I (control inhibitor 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 activator IFN-gamma at the pre-determined EC80 concentration is added to wells containing the control inhibitor or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

JAK/STAT - SIE-bla ME-180 - Activator Screen
SIE-bla ME-180 cells are thawed and resuspended in Assay Media (OPTI-MEM, 0.5% dialyzed FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 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 IL-6 (control activator starting concentration, 20 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

JAK/STAT - SIE-bla ME-180 - Inhibitor Screen, Activated by IL-6
SIE-bla ME-180 cells are thawed and prepared as described above for the Activator 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 JAK Inhibitor I (control inhibitor 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 activator IL-6 at the pre-determined EC80 concentration is added to wells containing the control inhibitor or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

JAK2/STAT5 - irf1-bla TF1 - Activator Screen
irf1-bla TF1 cells are thawed and resuspended in Assay Media (OPTI-MEM, 0.5% dialyzed FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 1,562,500 cells/mL. 32 µL of cell suspension (50,000 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 EPO (control activator 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

JAK2/STAT5 - irf1-bla TF1 - Inhibitor Screen, Activated by EPO
irf1-bla TF1 cells are thawed and prepared as described above for the Activator 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 JAK Inhibitor I (control inhibitor 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 activator EPO at the pre-determined EC80 concentration is added to wells containing the control inhibitor or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

JAK2/STAT5 - irf1-bla TF1 - Activator Screen
irf1-bla TF1 cells are thawed and resuspended in Assay Media (OPTI-MEM, 0.5% dialyzed FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 1,562,500 cells/mL. 32 µL of cell suspension (50,000 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 GM-CSF (control activator starting concentration, 0.25 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

JAK2/STAT5 - irf1-bla TF1 - Inhibitor Screen, Activated by GM-CSF
irf1-bla TF1 cells are thawed and prepared as described above for the Activator 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 JAK Inhibitor I (control inhibitor 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 activator GM-CSF at the pre-determined EC80 concentration is added to wells containing the control inhibitor or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

MAPK - c-fos-bla HEK 293T - Activator Screen
c-fos-bla HEK 293T cells are thawed and resuspended in Assay Media (OPTI-MEM, 0.5% dialyzed FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 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 PMA (control activator 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

MAPK - c-fos-bla HEK 293T - Inhibitor Screen, Activated by PMA
c-fos-bla HEK 293T cells are thawed and prepared as described above for the Activator 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 U0126 (control inhibitor 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 activator PMA at the pre-determined EC80 concentration is added to wells containing the control inhibitor or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

MAPK - c-fos-bla ME-180 - Activator Screen
c-fos-bla ME-180 cells are thawed and resuspended in Assay Media (OPTI-MEM, 0.5% dialyzed FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 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 EGF (control activator 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

MAPK - c-fos-bla ME-180 - Inhibitor Screen, Activated by EGF
c-fos-bla ME-180 cells are thawed and prepared as described above for the Activator 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 PD153035 (control inhibitor 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 activator EGF at the pre-determined EC80 concentration is added to wells containing the control inhibitor or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

MAPK/EGFR/Ras/Raf - AP1-bla ME-180 - Activator Screen
AP1-bla ME-180 cells are thawed and resuspended in Assay Media (OPTI-MEM, 0.5% dialyzed FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 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 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 EGF (control activator starting concentration, 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

MAPK/EGFR/Ras/Raf - AP1-bla ME-180 - Inhibitor Screen, Activated by EGF
AP1-bla ME-180 cells are thawed and prepared as described above for the Activator 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 PD153035 (control inhibitor 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 activator EGF at the pre-determined EC80 concentration is added to wells containing the control inhibitor or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

MAPK/MEK/B-raf - AP1-bla A375 - Inhibitor Screen, Constitutively Activated
AP1-bla A375 cells are thawed and resuspended in Assay Media (OPTI-MEM, 0.5% dialyzed FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 4 µL of a 10X serial dilution of Raf1 Kinase Inhibitor (control inhibitor starting concentration, 10,000 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

NFKB - NFKB-bla Jurkat - Activator Screen
NFKB-bla Jurkat 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. 4 µL of a 10X serial dilution of TNF-alpha (control activator starting concentration, 3 nM) or compounds are added to appropriate wells of a 384-well TC-Treated assay plate. 32 µL of cell suspension (20,000 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 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

NFKB - NFKB-bla Jurkat - Inhibitor Screen, Activated by TNF-alpha
NFKB-bla Jurkat cells are thawed and prepared as described above for the Activator Screen. 4 µL of a 10X serial dilution of Withaferin A (control inhibitor 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 inhibitor titration for 30 minutes. 4 µL of 10X control activator TNF-alpha at the pre-determined EC80 concentration is added to wells containing the control inhibitor or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

NFkB - NFkB-bla ME-180 - Activator Screen
NFkB-bla ME-180 cells are thawed and resuspended in Assay Media (OPTI-MEM, 0.5% dialyzed FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 375,000 cells/mL. 32 µL of cell suspension (12,000 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-1 beta (control activator 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

NFkB - NFkB-bla ME-180 - Inhibitor Screen, Activated by IL-1 beta
NFkB-bla ME-180 cells are thawed and prepared as described above for the Activator 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 Withaferin A (control inhibitor 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 activator IL-1 beta at the pre-determined EC80 concentration is added to wells containing the control inhibitor or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

NFkB - NFkB-bla ME-180 - Activator Screen
NFkB-bla ME-180 cells are thawed and resuspended in Assay Media (OPTI-MEM, 0.5% dialyzed FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 375,000 cells/mL. 32 µL of cell suspension (12,000 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 TNF-alpha (control activator 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

NFkB - NFkB-bla ME-180 - Inhibitor Screen, Activated by TNF-alpha
NFkB-bla ME-180 cells are thawed and prepared as described above for the Activator 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 Withaferin A (control inhibitor 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 activator TNF-alpha at the pre-determined EC80 concentration is added to wells containing the control inhibitor or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

NFkB - NFkB-bla THP-1 - Activator Screen
NFkB-bla THP-1 cells are thawed and resuspended in Assay Media (RPMI, 10% dialyzed FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 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 TNF-alpha (control activator 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

NFkB - NFkB-bla THP-1 - Inhibitor Screen, Activated by TNF-alpha
NFkB-bla THP-1 cells are thawed and prepared as described above for the Activator 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 Withaferin A (control inhibitor 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 activator TNF-alpha at the pre-determined EC80 concentration is added to wells containing the control inhibitor or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

Oxidative Stress - ARE-bla HepG2 - Activator Screen
ARE-bla HepG2 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 375,000 cells/mL. 32 µL of cell suspension (12,000 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 tBHQ (control activator starting concentration, 150,000 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.

Oxidative Stress - ARE-bla HepG2 - Inhibitor Screen, Activated by tBHQ
ARE-bla HepG2 cells are thawed and prepared as described above for the Activator 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 Ro-31-8220 (control inhibitor 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 activator tBHQ at the pre-determined EC80 concentration is added to wells containing the control inhibitor 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.

PI3K/AKT/FOXO3 - TREx FOXO3-DBE-bla HeLa - Activator Screen
TREx FOXO3-DBE-bla HeLa cells are thawed and resuspended in Assay Media (DMEM, 0.1% dialyzed FBS, 0.1 mM NEAA, 25 mM HEPES pH 7.3, 100 U/mL/100 ug/mL Pen/Strep, 5 ng/ml Doxycycline) to a concentration of 312,500 cells/mL. 4 µL of a 10X serial dilution of Insulin (control activator starting concentration, 1,000 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 Loading Solution is added to each well and the plate is incubated for 3 hours at room temperature. The plate is read on a fluorescence plate reader.

PI3K/AKT/FOXO3 - TREx FOXO3-DBE-bla HeLa - Inhibitor Screen, Activated by Insulin
TREx FOXO3-DBE-bla HeLa cells are thawed and prepared as described above for the Activator Screen. 4 µL of a 10X serial dilution of Triciribine (control inhibitor 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 inhibitor titration for 30 minutes. 4 µL of 10X control activator Insulin at the pre-determined EC80 concentration is added to wells containing the control inhibitor or compounds. The plate is incubated for 16-24 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 3 hours at room temperature. The plate is read on a fluorescence plate reader.

PKC/Ca2+ - NFAT-bla Jurkat - Activator Screen
NFAT-bla Jurkat cells are thawed and resuspended in Assay Media (OPTI-MEM, 0.5% dialyzed FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 781,250 cells/mL. 4 µL of a 10X serial dilution of Thapsigargin (control activator starting concentration, 100 nM) or compounds are added to appropriate wells of a 384-well TC-Treated assay plate. 32 µL of cell suspension (25,000 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 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

PKC/Ca2+ - NFAT-bla Jurkat - Inhibitor Screen, Activated by Thapsigargin
NFAT-bla Jurkat cells are thawed and prepared as described above for the Activator Screen. 4 µL of a 10X serial dilution of Ro-31-8220 (control inhibitor 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 inhibitor titration for 30 minutes. 4 µL of 10X control activator Thapsigargin at the pre-determined EC80 concentration is added to wells containing the control inhibitor or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

PKC/Ca2+ - TrkA-NFAT-bla CHO-K1 - Activator Screen
TrkA-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 0.1% dialyzed FBS, 0.1 mM NEAA, 25 mM HEPES pH 7.3, 100 U/mL/100 ug/mL Pen/Strep) to a concentration of 312,500 cells/mL. 4 µL of a 10X serial dilution of NGF 2.5S (control activator starting concentration, 38.5 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 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.

PKC/Ca2+ - TrkA-NFAT-bla CHO-K1 - Inhibitor Screen, Activated by NGF 2.5S
TrkA-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Activator Screen. 4 µL of a 10X serial dilution of K252a (control inhibitor starting concentration, 100 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 inhibitor titration for 30 minutes. 4 µL of 10X control activator NGF 2.5S at the pre-determined EC80 concentration is added to wells containing the control inhibitor 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.

PKC/Ca2+ - TrkB-NFAT-bla CHO-K1 - Activator Screen
TrkB-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 0.1% dialyzed FBS, 0.1 mM NEAA, 25 mM HEPES pH 7.3, 100 U/mL/100 ug/mL Pen/Strep) to a concentration of 312,500 cells/mL. 4 µL of a 10X serial dilution of BDNF (control activator starting concentration, 50 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 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.

PKC/Ca2+ - TrkB-NFAT-bla CHO-K1 - Inhibitor Screen, Activated by BDNF
TrkB-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Activator Screen. 4 µL of a 10X serial dilution of K252a (control inhibitor starting concentration, 100 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 inhibitor titration for 30 minutes. 4 µL of 10X control activator BDNF at the pre-determined EC80 concentration is added to wells containing the control inhibitor 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.

PKC/Ca2+ - TrkC-NFAT-bla CHO-K1 - Activator Screen
TrkC-NFAT-bla CHO-K1 cells are thawed and resuspended in Assay Media (DMEM, 0.1% dialyzed FBS, 0.1 mM NEAA, 25 mM HEPES pH 7.3, 100 U/mL/100 ug/mL Pen/Strep) to a concentration of 312,500 cells/mL. 4 µL of a 10X serial dilution of Neurotrophin-3 (control activator starting concentration, 35.7 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 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.

PKC/Ca2+ - TrkC-NFAT-bla CHO-K1 - Inhibitor Screen, Activated by Neurotrophin-3
TrkC-NFAT-bla CHO-K1 cells are thawed and prepared as described above for the Activator Screen. 4 µL of a 10X serial dilution of K252a (control inhibitor starting concentration, 100 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 inhibitor titration for 30 minutes. 4 µL of 10X control activator Neurotrophin-3 at the pre-determined EC80 concentration is added to wells containing the control inhibitor 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.

T cell receptor - NFAT-bla Jurkat - Activator Screen
NFAT-bla Jurkat cells are thawed and resuspended in Assay Media (OPTI-MEM, 0.5% dialyzed FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 781,250 cells/mL. 4 µL of a 10X serial dilution of anti-CD3:CD28 * (control activator starting concentration, 4 nM) or compounds are added to appropriate wells of a 384-well TC-Treated assay plate. 32 µL of cell suspension (25,000 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 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

T cell receptor - NFAT-bla Jurkat - Inhibitor Screen, Activated by anti-CD3:CD28 *
NFAT-bla Jurkat cells are thawed and prepared as described above for the Activator Screen. 4 µL of a 10X serial dilution of Ro-31-8220 (control inhibitor 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 inhibitor titration for 30 minutes. 4 µL of 10X control activator anti-CD3:CD28 * at the pre-determined EC80 concentration is added to wells containing the control inhibitor or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

TGF-beta - SBE-bla HEK 293T - Activator Screen
SBE-bla HEK 293T cells are thawed and resuspended in Assay Media (OPTI-MEM, 0.5% dialyzed FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 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 TGF-beta 1 (control activator starting concentration, 0.1 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

TGF-beta - SBE-bla HEK 293T - Inhibitor Screen, Activated by TGF-beta 1
SBE-bla HEK 293T cells are thawed and prepared as described above for the Activator 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 TGF-beta R1 (control inhibitor 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 activator TGF-beta 1 at the pre-determined EC80 concentration is added to wells containing the control inhibitor or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

TNF-alpha/JNK - AP1-bla ME-180 - Activator Screen
AP1-bla ME-180 cells are thawed and resuspended in Assay Media (OPTI-MEM, 0.5% dialyzed FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 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 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 TNF-alpha (control activator starting concentration, 50 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

TNF-alpha/JNK - AP1-bla ME-180 - Inhibitor Screen, Activated by TNF-alpha
AP1-bla ME-180 cells are thawed and prepared as described above for the Activator 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 JNK Inhibitor II (control inhibitor 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 activator TNF-alpha at the pre-determined EC80 concentration is added to wells containing the control inhibitor or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

Toll-like Receptor (TLR4) - NFkB-bla THP-1 - Activator Screen
NFkB-bla THP-1 cells are thawed and resuspended in Assay Media (RPMI, 0.5% dialyzed FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 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 LPS (control activator 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

Toll-like Receptor (TLR4) - NFkB-bla THP-1 - Inhibitor Screen, Activated by LPS
NFkB-bla THP-1 cells are thawed and prepared as described above for the Activator 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 Withaferin A (control inhibitor 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 activator LPS at the pre-determined EC80 concentration is added to wells containing the control inhibitor or compounds. The plate is incubated for 5 hours at 37ºC/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

Wnt/Beta-Catenin - LEF-TCF-bla HCT116 - Activator Screen, Constitutively Activated, further activated with Mouse Rec. Wnt-3A
LEF-TCF-bla HCT116 cells are thawed and resuspended in Assay Media (OPTI-MEM, 0.5% dialyzed FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 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 Mouse Rec. Wnt-3A (control activator 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

Wnt/Beta-Catenin - LEF-TCF-bla HCT116 - Inhibitor Screen, Constitutively Activated
LEF-TCF-bla HCT116 cells are thawed and prepared as described above for the Activator 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 ICG-001 (control inhibitor 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

Wnt/Beta-Catenin (APC-/-) - LEF-TCF-bla SW480 - Inhibitor Screen, Constitutively Activated
LEF-TCF-bla SW480 cells are thawed and resuspended in Assay Media (OPTI-MEM, 0.5% dialyzed FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 4 µL of a 10X serial dilution of ICG-001 (control inhibitor starting concentration, 10,000 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.