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Screening Protocol and Assay Conditions

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Overview and Assay Theory

The SelectScreen Cell-Based Nuclear Receptor Profiling Service uses the GeneBLAzer™ Beta-lactamase reporter technology to provide you with a reliable, rapid and sensitive method for analyzing the intracellular effect(s) of test compounds on several important Nuclear Receptors (NRs). These functional assays allow for measurement of receptor agonism or antagonism by compounds and can be used to determine compound potency and selectivity.

All GeneBLAzer Validated Assays used in the SelectScreen Cell-Based Nuclear Receptor Profiling Service utilize division arrested cells to eliminate variability associated with cell division and provides superior response profiles as a result of response element optimization and use of optimal clones.

GeneBLAzer Validated Assays for NRs:

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

The GeneBLAzer Validated Assays 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 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.

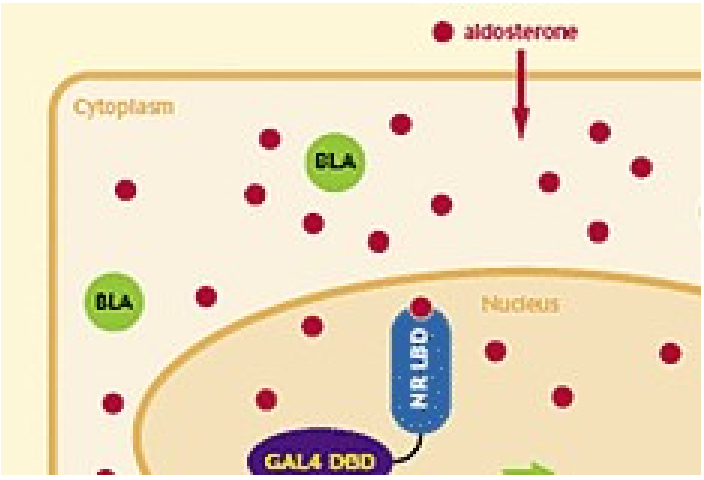

Figure 1: GeneBLAzer technology. Nuclear Receptor Assays use a Beta-lactamase cDNA under transcriptional control of an Upstream Activator Sequence (UAS). The UAS is activated by the GAL4 transcription factor DNA binding domain (DBD), which is expressed as a fusion protein with the target receptor ligand binding domain (LBD). Upon ligand binding (aldosterone, in this example), the GAL4(DBD)-NR(LBD), binds to the UAS, which controls transcription of Beta -lactamase.

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, in the presence of *bla* expression, 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.

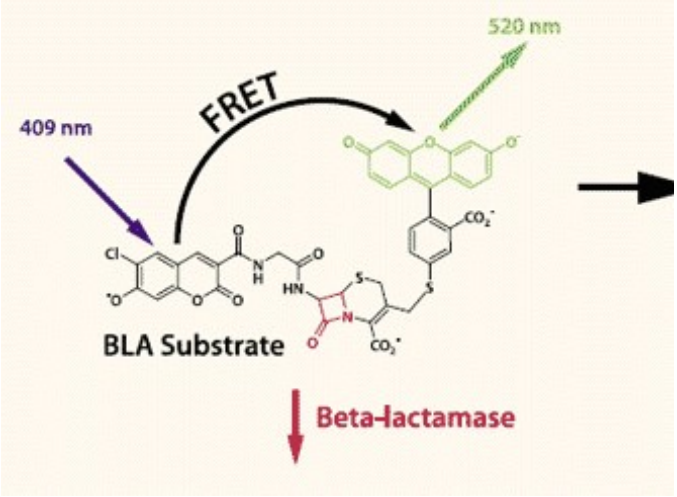

Figure 2: Fluorescent detection of cells using the GeneBLAzer™ technology. After substrate loading, in the absence of Beta-lactamase, cells appear green. In the presence of Beta-lactamase, the substrate is cleaved and cells appear blue.

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 that 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 Life Technologies reagents: Solution A (10 mM LiveBLAzer™-FRET B/G Substrate), Solution B and Solution C.

Agonist Assay Protocol

Plate type utilized is dictated by each cell line as 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. 40 nL of 1000X compound or known activator titration plus 4 µL of assay media is added to the assay plate.
2. 32 µL of cells diluted in Assay Media to appropriate cell density are added to 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 16-24 hours (depending upon the assay 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 agonist to add in step 3.

1. 40 nL of 1000X compound or known activator titration plus 4 µL of assay media is added to the assay plate.
2. 32 µL of cells diluted in Assay Media to appropriate cell density are added to the assay plate and incubated for 30-60 minutes at 37ºC/5% CO_2 in a humidified incubator.
3. 4 µL of the 10X EC_{80} concentration of stim (agonist), as determined in an Agonist assay, is added to all wells containing test compound and known antagonist to bring the final assay volume to 40 µL.
4. 4 µL of Assay Medium is added to remaining control wells to bring their volume up to 40 µL.
5. The assay plate is incubated for 16-24 hours (depending upon the assay 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 nuclear receptor cell line:

Full Stim Control

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

No Stim Control

The no stim control contains 0.1% DMSO, cells and assay media in the place of stim (agonist). 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. These values are 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 mode 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 Inhibitor (Antagonist Mode) Titration

A known stim (agonist) or inhibitor titration is run on every assay plate for each cell line to ensure the cell line is either stimulated 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	$\dfrac{\textbf{Emission Ratio}_{\text{Compound}}}{\textbf{Emission Ratio}_{\text{No Stim Ctrl}}}$
% Activation – Agonist Assays	$\left\{\dfrac{\textbf{Response Ratio}_{\text{Compound}} - \textbf{Response Ratio}_{\text{No Stim Ctrl}}}{\textbf{Response Ratio}_{\text{Full Stim Ctrl}} - \textbf{Response Ratio}_{\text{No Stim Ctrl}}}\right\} * 100$
% Inhibition – Antagonist Assays	$\left\{1 - \dfrac{\textbf{Response Ratio}_{\text{Compound}} - \textbf{Response Ratio}_{\text{No Stim Ctrl}}}{\textbf{Response Ratio}_{\text{EC80Ctrl}} - \textbf{Response Ratio}_{\text{No Stim Ctrl}}}\right\} * 100$
Z´ - Agonist Assays (using Emission Ratio values)	$1 - \dfrac{\textbf{3*Std Dev}_{\text{Full Stim Ctrl}} + \textbf{3*Std Dev}_{\text{No Stim Ctrl}}}{\textbf{Mean}_{\text{Full Stim Ctrl}} - \textbf{Mean}_{\text{No Stim Ctrl}}}$
Z´ - Antagonist Assays (using Emission Ratio values)	$1 - \dfrac{\textbf{3*Std Dev}_{\text{EC80 Ctrl}} + \textbf{3*Std Dev}_{\text{No Stim Ctrl}}}{\textbf{Mean}_{\text{EC80 Ctrl}} - \textbf{Mean}_{\text{No Stim Ctrl}}}$

Graphing Software

SelectScreen Cell-Based Nuclear Receptor Profiling Service uses XL*fit* from IDBS. The dose response curve is curve fit to model number 205 (sigmoidal dose-response model).

Nuclear Receptor Cell Lines Available for Screening

Assay	Cell Line	Tech-nology	Agonist	EC50 (nM)	Antagonist	IC50 (nM)	Ag. Mode	Antag. Mode
AR	UAS-bla GripTite™ 293	BLA	R1881	0.322	Cyproterone Acetate	77.43	Yes	Yes
ER-alpha	UAS-bla GripTite™ 293	BLA	17-beta-Estradiol	0.378	4-hydroxytamoxifen	2.608	Yes	Yes
ER-beta	UAS-bla GripTite™ 293	BLA	17-beta-Estradiol	3.688	RU-486	100.7	Yes	Yes
ERR-alpha	UAS-bla HEK 293T	BLA	None	---	XCT790	322.8		Yes
FXR	UAS-bla HEK 293T	BLA	GW4064	44.0	None	---	Yes	Yes
GR	UAS-bla HEK 293T	BLA	Dexamethasone	2.966	RU-486	0.795	Yes	Yes
LXR-alpha	UAS-bla HEK 293T	BLA	TO901317	1.662	None	---	Yes	Yes
LXR-beta	UAS-bla HEK 293T	BLA	TO901317	11.1	None	---	Yes	Yes
MR	UAS-bla HEK 293T	BLA	Aldosterone	0.402	Spironolactone	6.22	Yes	Yes
PPAR-delta	UAS-bla HEK 293T	BLA	L-165041	19.3	MK-886	14,320	Yes	Yes
PPAR-gamma	UAS-bla HEK 293H	BLA	Rosiglitazone	4.86	T0070907	15.5	Yes	Yes
PR	UAS-bla HEK 293T	BLA	R5020	2.069	RU-486	0.818	Yes	Yes
RAR-alpha	UAS-bla HEK 293T	BLA	ATRA	0.780	Ro-41-5253	124.6	Yes	Yes
RAR-beta	UAS-bla HEK 293T	BLA	ATRA	0.646	AGN193109	3.64	Yes	Yes
RAR-gamma	UAS-bla HEK 293T	BLA	ATRA	0.196	AGN193109	0.473	Yes	Yes
RXR-alpha	UAS-bla HEK 293T	BLA	9-cis retinoic acid	266.1	None	---	Yes	Yes
RXR-beta	UAS-bla HEK 293T	BLA	9-cis retinoic acid	275.2	None	---	Yes	Yes
TR-alpha	UAS-bla HEK 293T	BLA	T3 Sodium Salt	0.102	Thyroid Hormone receptor antagonist 1-850	18,700	Yes	Yes
TR-beta	UAS-bla HEK 293T	BLA	T3 Free Acid	0.315	None	---	Yes	Yes
VDR	UAS-bla HEK 293T	BLA	Calcitriol	0.105	None	---	Yes	Yes

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

Nuclear Receptor Cell Line-Specific Assay Conditions

AR - Agonist Screen
AR-UAS-bla GripTite™ 293 cells are thawed and resuspended in Assay Media (DMEM phenol red free, 2% CD-treated 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 R1881 (control agonist starting concentration, 31.6 nM) or compounds are added to appropriate wells of a 384-well Poly-D-Lysine 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 2 hours at room temperature. The plate is read on a fluorescence plate reader.

AR - Antagonist Screen, Activated by R1881
AR-UAS-bla GripTite™ 293 cells are thawed and prepared as described above for the Agonist Screen. 4 µL of a 10X serial dilution of Cyproterone Acetate (control antagonist starting concentration, 3,160 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 antagonist titration for 30 minutes. 4 µL of 10X control agonist R1881 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 Loading Solution is 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-alpha - Agonist Screen
ER-alpha-UAS-bla GripTite™ 293 cells are thawed and resuspended in Assay Media (DMEM phenol red free, 2% CD-treated 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 17-beta-Estradiol (control agonist starting concentration, 10 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 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-alpha - Antagonist Screen, Activated by 17-beta-Estradiol
ER-alpha-UAS-bla GripTite™ 293 cells are thawed and prepared as described above for the Agonist Screen. 4 µL of a 10X serial dilution of 4-hydroxytamoxifen (control antagonist 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 antagonist titration for 30 minutes. 4 µL of 10X control agonist 17-beta-Estradiol 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 Loading Solution is 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-beta - Agonist Screen
ER-beta-UAS-bla GripTite™ 293 cells are thawed and resuspended in Assay Media (DMEM phenol red free, 2% CD-treated 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 17-beta-Estradiol (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 (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 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-beta - Antagonist Screen, Activated by 17-beta-Estradiol
ER-beta-UAS-bla GripTite™ 293 cells are thawed and prepared as described above for the Agonist Screen. 4 µL of a 10X serial dilution of RU-486 (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 17-beta-Estradiol 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

ERR-alpha - Antagonist Screen, Activated by None
ERR-alpha-UAS-bla HEK 293T cells are thawed and resuspended in Assay Media (DMEM phenol red free, 2% CD-treated 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 XCT790 (control antagonist starting concentration, 5,000 nM) or compounds are added to appropriate wells of a Poly-D-Lysine assay plate. 32 µL of cell suspension (20,000 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.

FXR - Agonist Screen
FXR-UAS-bla HEK 293T cells are thawed and resuspended in Assay Media (DMEM phenol red free, 2% CD-treated 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 GW4064 (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 (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 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.

FXR - Antagonist Screen, Activated by GW4064
FXR-UAS-bla HEK 293T 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 GW4064 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 Loading Solution is 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 FXR-UAS-bla HEK 293T assay does not have an antagonist control.

GR - Agonist Screen
GR-UAS-bla HEK 293T cells are thawed and resuspended in Assay Media (DMEM phenol red free, 2% CD-treated 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 Dexamethasone (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 (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 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.

GR - Antagonist Screen, Activated by Dexamethasone
GR-UAS-bla HEK 293T cells are thawed and prepared as described above for the Agonist Screen. 4 µL of a 10X serial dilution of RU-486 (control antagonist starting concentration, 10 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 Dexamethasone 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

LXR-alpha - Agonist Screen
LXR-alpha-UAS-bla HEK 293T cells are thawed and resuspended in Assay Media (DMEM phenol red free, 2% CD-treated 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 TO901317 (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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

LXR-alpha - Antagonist Screen, Activated by TO901317
LXR-alpha-UAS-bla HEK 293T 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 TO901317 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 Loading Solution is 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 LXR-alpha-UAS-bla HEK 293T assay does not have an antagonist control.

LXR-beta - Agonist Screen
LXR-beta-UAS-bla HEK 293T cells are thawed and resuspended in Assay Media (DMEM phenol red free, 2% CD-treated 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 TO901317 (control agonist starting concentration, 3,000 nM) or compounds are added to appropriate wells of a 384-well Poly-D-Lysine 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 2 hours at room temperature. The plate is read on a fluorescence plate reader.

LXR-beta - Antagonist Screen, Activated by TO901317
LXR-beta-UAS-bla HEK 293T 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 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 antagonist titration for 30 minutes. 4 µL of 10X control agonist TO901317 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 Loading Solution is 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 LXR-beta-UAS-bla HEK 293T assay does not have an antagonist control.

MR - Agonist Screen
MR-UAS-bla HEK 293T cells are thawed and resuspended in Assay Media (DMEM phenol red free, 2% CD-treated 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 Aldosterone (control agonist starting concentration, 100 nM) or compounds are added to appropriate wells of a 384-well Poly-D-Lysine 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 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.

MR - Antagonist Screen, Activated by Aldosterone
MR-UAS-bla HEK 293T cells are thawed and prepared as described above for the Agonist Screen. 4 µL of a 10X serial dilution of Spironolactone (control antagonist starting concentration, 1,000 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 antagonist titration for 30 minutes. 4 µL of 10X control agonist Aldosterone 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

PPAR-delta - Agonist Screen
PPAR-delta-UAS-bla HEK 293T cells are thawed and resuspended in Assay Media (DMEM phenol red free, 2% CD-treated 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 L-165041 (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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

PPAR-delta - Antagonist Screen, Activated by L-165041
PPAR-delta-UAS-bla HEK 293T cells are thawed and prepared as described above for the Agonist Screen. 4 µL of a 10X serial dilution of MK-886 (control antagonist starting concentration, 20,000 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 L-165041 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

PPAR-gamma - Agonist Screen
PPAR-gamma-UAS-bla HEK 293H cells are thawed and resuspended in Assay Media (DMEM phenol red free, 2% CD-treated FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 937,500 cells/mL. 4 µL of a 10X serial dilution of Rosiglitazone (control agonist starting concentration, 316 nM) or compounds are added to appropriate wells of a 384-well Poly-D-Lysine assay plate. 32 µL of cell suspension (30,000 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 2 hours at room temperature. The plate is read on a fluorescence plate reader.

PPAR-gamma - Antagonist Screen, Activated by Rosiglitazone
PPAR-gamma-UAS-bla HEK 293H cells are thawed and prepared as described above for the Agonist Screen. 4 µL of a 10X serial dilution of T0070907 (control antagonist starting concentration, 1,000 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 antagonist titration for 30 minutes. 4 µL of 10X control agonist Rosiglitazone 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

PR - Agonist Screen
PR-UAS-bla HEK 293T cells are thawed and resuspended in Assay Media (DMEM phenol red free, 2% CD-treated FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 468,750 cells/mL. 4 µL of a 10X serial dilution of R5020 (control agonist starting concentration, 100 nM) or compounds are added to appropriate wells of a 384-well Poly-D-Lysine assay plate. 32 µL of cell suspension (15,000 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 2 hours at room temperature. The plate is read on a fluorescence plate reader.

PR - Antagonist Screen, Activated by R5020
PR-UAS-bla HEK 293T cells are thawed and prepared as described above for the Agonist Screen. 4 µL of a 10X serial dilution of RU-486 (control antagonist 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 antagonist titration for 30 minutes. 4 µL of 10X control agonist R5020 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

RAR-alpha - Agonist Screen
RAR-alpha-UAS-bla HEK 293T cells are thawed and resuspended in Assay Media (DMEM phenol red free, 2% CD-treated 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 ATRA (control agonist starting concentration, 10 nM) or compounds are added to appropriate wells of a 384-well Poly-D-Lysine 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 2 hours at room temperature. The plate is read on a fluorescence plate reader.

RAR-alpha - Antagonist Screen, Activated by ATRA
RAR-alpha-UAS-bla HEK 293T cells are thawed and prepared as described above for the Agonist Screen. 4 µL of a 10X serial dilution of Ro-41-5253 (control antagonist starting concentration, 500 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 antagonist titration for 30 minutes. 4 µL of 10X control agonist ATRA 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

RAR-beta - Agonist Screen
RAR-beta-UAS-bla HEK 293T cells are thawed and resuspended in Assay Media (DMEM phenol red free, 0.1% BSA, 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 ATRA (control agonist starting concentration, 10 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 2 hours at room temperature. The plate is read on a fluorescence plate reader.

RAR-beta - Antagonist Screen, Activated by ATRA
RAR-beta-UAS-bla HEK 293T cells are thawed and prepared as described above for the Agonist Screen. 4 µL of a 10X serial dilution of AGN193109 (control antagonist starting concentration, 10 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 ATRA 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

RAR-gamma - Agonist Screen
RAR-gamma-UAS-bla HEK 293T cells are thawed and resuspended in Assay Media (DMEM phenol red free, 2% CD-treated 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. 4 µL of a 10X serial dilution of ATRA (control agonist starting concentration, 10 nM) or compounds are added to appropriate wells of a 384-well TC-Treated assay plate. 32 µL of cell suspension (5,000 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 2 hours at room temperature. The plate is read on a fluorescence plate reader.

RAR-gamma - Antagonist Screen, Activated by ATRA
RAR-gamma-UAS-bla HEK 293T cells are thawed and prepared as described above for the Agonist Screen. 4 µL of a 10X serial dilution of AGN193109 (control antagonist starting concentration, 10 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 ATRA 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 Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader.

RXR-alpha - Agonist Screen

RXR-alpha-UAS-bla HEK 293T cells are thawed and resuspended in Assay Media (DMEM phenol red free, 2% CD-treated 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 9-cis retinoic acid (control agonist 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 2 hours at room temperature. The plate is read on a fluorescence plate reader.

RXR-alpha - Antagonist Screen, Activated by 9-cis retinoic acid
RXR-alpha-UAS-bla HEK 293T 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 9-cis retinoic acid 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 Loading Solution is 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 RXR-alpha-UAS-bla HEK 293T assay does not have an antagonist control.

RXR-beta - Agonist Screen
RXR-beta-UAS-bla HEK 293T cells are thawed and resuspended in Assay Media (DMEM phenol red free, 2% CD-treated 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 9-cis retinoic acid (control agonist 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 2 hours at room temperature. The plate is read on a fluorescence plate reader.

RXR-beta - Antagonist Screen, Activated by 9-cis retinoic acid
RXR-beta-UAS-bla HEK 293T 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 9-cis retinoic acid 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 Loading Solution is 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 RXR-beta-UAS-bla HEK 293T assay does not have an antagonist control.

TR-alpha - Agonist Screen
TR-alpha-UAS-bla HEK 293T cells are thawed and resuspended in Assay Media (DMEM phenol red free, 2% CD-treated 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 T3 Sodium Salt (control agonist starting concentration, 4 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 22-24 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.

TR-alpha - Antagonist Screen, Activated by T3 Sodium Salt
TR-alpha-UAS-bla HEK 293T cells are thawed and prepared as described above for the Agonist Screen. 4 µL of a 10X serial dilution of Thyroid Hormone receptor antagonist 1-850 (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 T3 Sodium Salt at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 22-24 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.

TR-beta - Agonist Screen
TR-beta-UAS-bla HEK 293T cells are thawed and resuspended in Assay Media (DMEM phenol red free, 2% CD-treated 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 T3 Free Acid (control agonist starting concentration, 10 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 2 hours at room temperature. The plate is read on a fluorescence plate reader.

TR-beta - Antagonist Screen, Activated by T3 Free Acid
TR-beta-UAS-bla HEK 293T 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 T3 Free Acid 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 Loading Solution is 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 TR-beta-UAS-bla HEK 293T assay does not have an antagonist control.

VDR - Agonist Screen
VDR-UAS-bla HEK 293T cells are thawed and resuspended in Assay Media (DMEM phenol red free, 2% CD-treated 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 Calcitriol (control agonist starting concentration, 50 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 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.

VDR - Antagonist Screen, Activated by Calcitriol
VDR-UAS-bla HEK 293T 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 Calcitriol 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 Loading Solution is 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 VDR-UAS-bla HEK 293T assay does not have an antagonist control.