Validation & Assay Performance Summary

GeneBLAzer® RXR-alpha DA Cells & Assay Kit

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GeneBLAzer® RXRα-UAS-*bla* HEK 293T Validated Assay

Cat. no. K1411, K1697

Target Description

The Retinoid X receptor-alpha (RXR alpha) is a nuclear hormone receptor and can function as a ligand inducible transcription factor capable of acting as a co-repressor and/or co-activator for gene expression. Nuclear receptors contain a series of conserved domains or regions. These domains/regions include a variable NH_2-domain (A/B region), a conserved DNA-binding domain (DBD or region C), a linker region (region D), a ligand binding domain (LBD or region E), and in some receptors a variable COOH-terminal (region F) (1). RXR alpha belongs to the family of retinoid X receptors, one of two retinoic receptor families (retinoic acid receptors and retinoid X receptors). RXR alpha is one of three members of the RXR family which consists of RXR alpha, RXR beta, and RXR gamma (11). The A/B and D regions of RXR alpha are involved in dictating the cell dependent transcriptional response (6).

RXR receptors are able to form both homo- and heterodimers (3). RXR receptors have been reported to form heterodimers with TRs (thyroid hormone receptors), RARs retinoic acid receptors), VDR (vitamin D receptor), PPARs (peroxisome proliferators activated receptor), LXR (liver X receptor), and FXR (farnesoid X receptor). These heterodimers can be classified as permissive and nonpermissive heterodimers (3). Addition of a RXR agonist, such as 9-cis-retinoic acid, can result in transcriptional activity of permissive heterodimers while activation of nonpermissive heterodimers occurs independent of the RXR agonist (3). RXR alpha agonist LG100268 activates the transcriptional response RXR:PPAR gamma and RXR:LXR heterodimers alone and synergistically with PPAR gamma and LXR agonists, but does not activate RXR:RAR and RXR:TR heterodimers whose activity is dependent upon RAR and TR agonists (10).

RXR alpha is expressed in the liver, spleen, placenta, epidermis, central nervous system, and is implicated in embryo development and differentiation (11). With its ability to form heterodimers with other nuclear receptors, RXR alpha has potential roles in lipid metabolism, skin alopecia, dermal cysts, cardiac development, insulin sensitization, and gene regulation. RXR activation has been shown in the spinal cord, brain, and epithelia of transgenic *Xenopus laevis* embryos. The development of a loss of function mutation of RXR alpha in a mouse germ line resulted in embryonic lethality due to defects in the ventricular walls of the heart (reviewed in 11).

The endogenous ligands for RXR alpha include 9-cis-retinoic acid (4,6), phytanic acid (12), and docosahexaenoic acid (7). Synthetic agonists for RXR alpha have been termed rexinoids and include LG100268 (10).

Cell Line Description

GeneBLAzer® RXR alpha DA(Division Arrested) cells and RXR alpha-UAS-*bla* HEK 293T cells contain the ligand-binding domain (LBD) of the human retinoid X receptor-alpha (RXR alpha) fused to the DNA-binding domain of GAL4 stably integrated in the GeneBLAzer® UAS-*bla* HEK 293T cell line. GeneBLAzer® UAS-*bla* HEK 293T cells stably express a beta-lactamase reporter gene under the transcriptional control of an upstream activator sequence (UAS). When an agonist binds to the LBD of the GAL4 (DBD)-RXR alpha (LBD) fusion protein, the protein binds to the UAS, resulting in expression of alpha-lactamase. Division Arrested (DA) cells are available in two configurations- an Assay Kit (which includes cells and sufficient substrate to analyze 1 x 384-well plate), and a tube of cells sufficient to analyze 10 x 384-well plates.

DA cells are irreversibly division arrested using a low-dose treatment of Mitomycin-C, and have no apparent toxicity or change in cellular signal transduction. Both RXR alpha DA cells and RXR alpha-UAS-*bla* HEK 293T cells are functionally validated for Z' and EC_{50} concentrations of 9-cis-retinoic acid (Figure 1). In addition, RXR alpha-UAS-*bla* HEK 293T cells have been tested for assay performance under variable conditions, including DMSO concentration, cell number, stimulation time, and substrate loading time (data available upon request). Additional testing data using alternate stimuli are also available.

Validation Summary

Performance of this assay was validated under optimized conditions in 384-well format using LiveBLAzer™-FRET B/G Substrate.

1. **Primary agonist dose response under optimized conditions (n=6)**

	DA	Dividing
9-cis-retinoic acid (EC_{50})	15nM	11nM
Z'-Factor (EC_{100})	0.91	0.79

Response Ratio = 6.9
Optimum cell no. = 10K cells/well
Optimum [DMSO] = up to 1%
Stimulation Time = 16 hours
Max. [Stimulation] = 10 µM

2. **Alternate agonist dose response**

All-trans-retinoic acid	EC_{50} = 4.1 nM
Phytanic acid	EC_{50} = 4.3 µM
Docosahexaenoic acid	EC_{50} = >10 µM

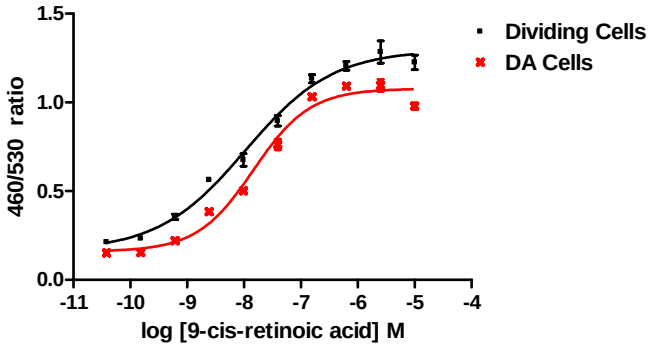
3. **Cell culture and maintenance**
See Cell Culture and Maintenance Section and Table 1

Assay Testing Summary

4. **Assay performance with variable cell number**
5. **Assay performance with variable stimulation time**
6. **Assay performance with variable substrate loading time**
7. **Assay performance with variable DMSO concentration**
8. **Alternate NR agonists dose response**
9. **Alternate NR agonists dose response (cont)**
10. **Alternate NR agonists in the presence and absence of RAR alpha antagonist RO41-5253 dose responses**
11. **Assay with variable cell number and plate type**

Primary Agonist Dose Response

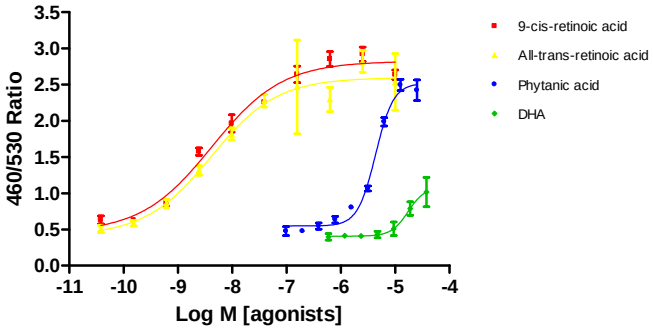
Figure 1 — RXR alpha DA and RXR alpha-UAS-*bla* HEK 293T dose response to 9-cis-retinoic acid under optimized conditions



RXR alpha DA cells and RXR alpha-UAS-*bla* HEK 293T cells (10,000 cells/well) were plated in a 384-well format and stimulated with a dilution series of 9-cis-retinoic acid in the presence of 0.5% DMSO for 16 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for 2 hours. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and plotted for each replicate against the concentrations of 9-cis-retinoic acid (n=6 for each data point).

Alternate Agonist Dose Response

Figure 2 —9-cis-retinoic acid, all-trans-retinoic acid, phytanic acid, and docosahexaenoic acid agonist dose Response



RXR alpha-UAS-*bla* HEK 293T cells (10,000 cells/well) were plated the day of the assay in a 384-well format. Cells were stimulated with either 9-cis-retinoic acid (Biomol #GR101), all-trans-retinoic acid (Sigma #R2500), phytanic acid (Sigma cat# P4060) and docosahexaenoic acid (DHA) (Sigma #D2534) over the indicated concentration range in the presence of 1.0% DMSO for 16 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for 90 minutes. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the Ratios plotted against the indicated concentrations of the agonists (n= 8 for each data point).

Dividing Cell Culture and Maintenance

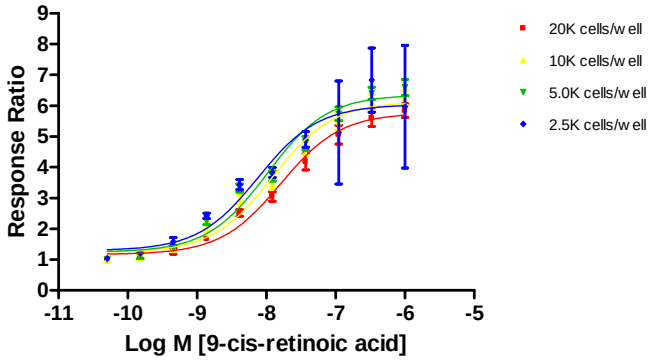
Dividing cells should be maintained at between 5 and 90% confluency in complete growth media and in a humidified incubator at 37ºC and 5% CO_2. Split dividing cells at least twice a week. Do not allow dividing cells to reach confluence.

Table 1 – Dividing Cell Culture and Maintenance

Component	Growth Medium (–)	Growth Medium (+)	Assay Medium	Freeze Medium
DMEM, w/ GlutaMAX™	90%	90%	—	—
Phenol Red free DMEM	—	—	98%	—
Dialyzed FBS **Do not substitute!**	10%	10%	—	—
Charcoal/Dextran FBS	—	—	2%	
NEAA	0.1 mM	0.1 mM	0.1 mM	—
HEPES (pH 7.3)	25 mM	25 mM	—	—
Hygromycin B	—	100 µg/mL	—	—
Zeocin™	—	100 µg/mL	—	—
Penicillin	100 U/mL	100 U/mL	100 U/mL	—
Streptomycin	100 µg/mL	100 µg/mL	100 µg/mL	—
Sodium Pyruvate	—	—	1 mM	
Recovery™ Cell Culture Freezing Medium	—	—	—	100%

Assay Performance with Variable Cell Number

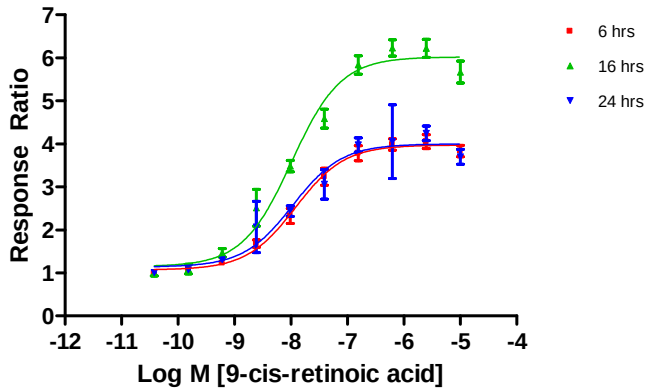
Figure 4— 9-cis-retinoic acid dose response with 2.5, 5.0, 10, and 20K cells/well



RXR alpha-UAS-*bla* HEK 293T cells were plated at 2,500, 5,000, 10,000 or 20,000 cells/well in a 384-well format the day of the assay. Cells were stimulated with 9-cis-retinoic acid (Biomol #GR101) for 18 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for 90 min. Fluorescence emission values at 460 nm and 530 nm for the various cell numbers were obtained using a standard fluorescence plate reader and the Response Ratios plotted against the indicated concentrations of 9-cis-retinoic acid (n=8 for each data point).

Assay performance with Variable Stimulation Time

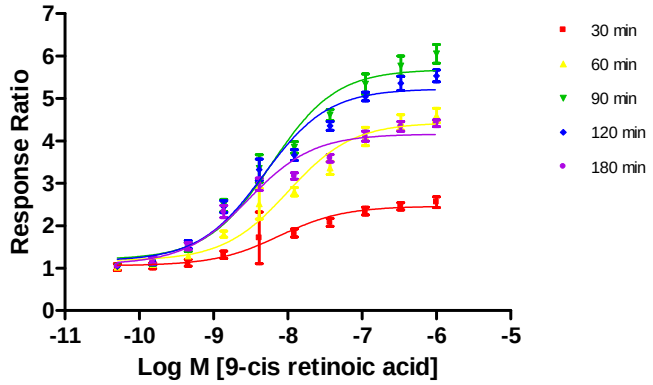
Figure 5 – Chenodeoxycholic acid dose response with 6, 16, and 24 hour stimulation times



RXR alpha-UAS-*bla* HEK 293T cells (10,000 cells/well) were plated the day of the assay in a 384-well poly-d-lysine treated black-walled tissue culture assay plate. Cells were stimulated with a serial dilution of 9-cis-retinoic acid (Biomol #GR101) for 6, 16, and 24 hours in 0.5% DMSO and then loaded for 90 minutes with LiveBLAzer™-FRET B/G Substrate(1µM final concentration of CCF4-AM). Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader. Response Ratios were plotted against the indicated concentrations of 9-cis-retinoic acid (n=16 for each data point)

Assay performance with Variable Substrate Loading Time

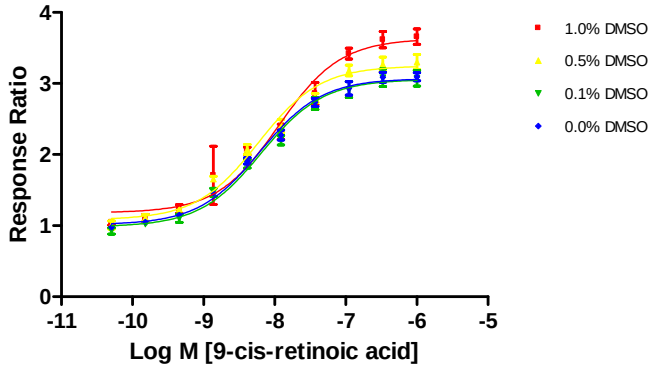
Figure 6 – 9-cis-retinoic acid dose response with 0.5, 1, 1.5, 2, and 3 hour loading times



RXR alpha-UAS-*bla* HEK 293T cells were plated at 10,000 cells/well in a 384-well format the day of the assay. Cells were stimulated with a dilution series of 9-cis-retinoic acid (Biomol #GR101) for 18 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for either 0.5, 1, 1.5, 2, or 3 hours. Fluorescence emission values at 460 nm and 530 nm for the various loading times were obtained using a standard fluorescence plate reader and the Response Ratios were plotted against the indicated concentrations of 9-cis-retinoic acid (n=16 for each data point).

Assay Performance with variable DMSO concentration

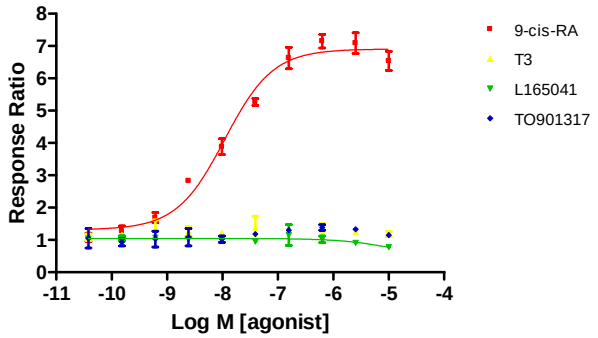
Figure 7 – 9-cis-Retinoic acid dose response with 0, 0.1, 0.5 and 1% DMSO.



RXR alpha-UAS-*bla* HEK 293T cells (10,000 cells/well) were plated the day of the assay in a 384-well black-walled tissue culture assay plate. DMSO was added to the cells at concentrations from 0% to 1%. Cells were stimulated with a serial dilution series of 9-cis-retinoic acid (Biomol cat# GR101) for 16 hours at 37ºC. Cells were loaded for 90 minutes with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM). Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the Response Ratios are shown plotted for each DMSO concentration against the indicated concentrations of 9-cis-retinoic acid (n=8 for each data point).

Dose Response with additional nuclear receptor agonists

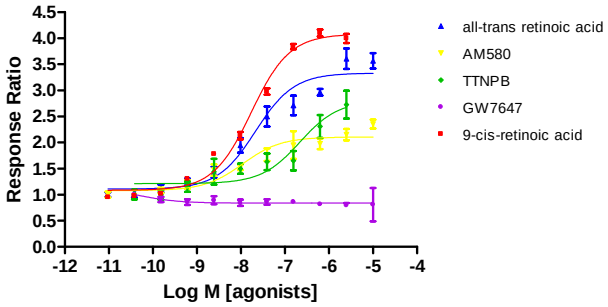
Figure 8 – Dose response of additional nuclear receptor agonists.



RXR alpha-UAS-*bla* HEK 293T cells (10,000 cells/well) were plated the day of the assay in a 384-well black-walled cellbind plate. Cells were stimulated with a serial dilution series of 9-cis-retinoic acid (Biomol cat# GR101), L165,041 (Sigma cat# L2167), TO901317 (Calbiochem cat# 575310), and T3 (thyroid hormone 3,5,3'-triiodothyronin) (Calbiochem cat# 642511) in the presence of 0.5% DMSO for 16 hours at 37ºC. Cells were loaded for 90 minutes with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM). Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the Response Ratios were plotted against the indicated concentrations of agonists (n=8 for each data point).

Dose Response with additional nuclear receptor agonists (cont)

Figure 9 – Dose response of additional nuclear receptor agonists.



RXR alpha-UAS-*bla* HEK 293T cells (10,000 cells/well) were plated the day of the assay in a 384-well black-walled cellbind plate. Cells were stimulated with a serial dilution series of 9-cis-retinoic acid (Biomol cat# GR101), all-trans-retinoic acid (Sigma cat# R2625), TTNPB (Sigma cat# T3757), GW7674 (Sigma cat# G6793), and AM580 (Sigma cat# A8843) in the presence of 0.5% DMSO for 16 hours at 37ºC. Cells were loaded for 90 minutes with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM). Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the Response Ratios were plotted against the indicated concentrations of agonists (n=8 for each data point).

Dose Response with additional nuclear receptor agonists in the presence and absence of RAR alpha antagonist RO41-5253

Figure 10a. – Dose response of 9-cis-retinoic acid in the presence or absence of RO41-5253

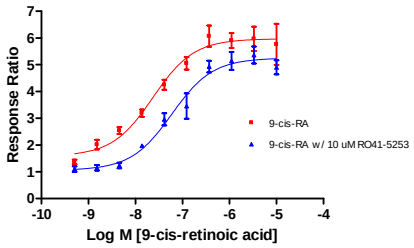


Figure 10b. – Dose response of all-trans-retinoic acid in the presence or absence of RO41-5253

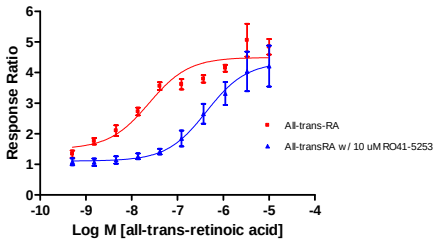


Figure 10c. – Dose response of RARα agonist AM580 in the presence or absence of RO41-5253

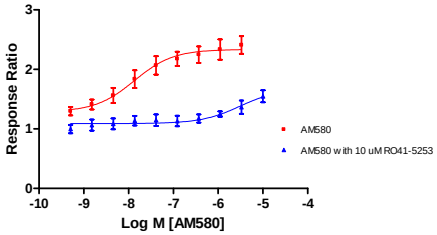
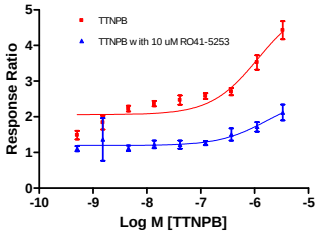


Figure 10d. – Dose response of TTNPB in the presence or absence of RO41-5253



RXR alpha-UAS-*bla* HEK 293T cells (10,000 cells/well) were plated the day of the assay in a 384-well black-walled cellbind plate. Cells were treated with 1% DMSO with and without 10 µM RO41-5253 (Biomol cat# G110) for 30 minutes at 37°C. Cells were stimulated with a serial dilution series of 9-cis-retinoic acid (Biomol cat# GR101), all-trans-retinoic acid (Sigma cat# R2625), TTNPB (Sigma cat# T3757), and AM580 (Sigma cat# A8843) for 16 hours at 37ºC. Cells were loaded for 90 minutes with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM). Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the Response Ratios were plotted against the indicated concentrations of agonists (n=8 for each data point).

Assay with variable cell number and plate type

Figure 11a. – 9-cis-retinoic acid dose response with 20K, 10K, 5.0K, and 2.5K cells/well in a poly-d-lysine (biocoat) plate.

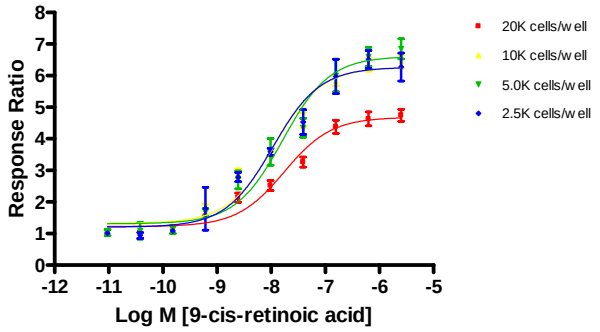


Figure 11b. – 9-cis-retinoic acid dose response with 20K, 10K, 5.0K, and 2.5K cells/well in a Cellbind plate.

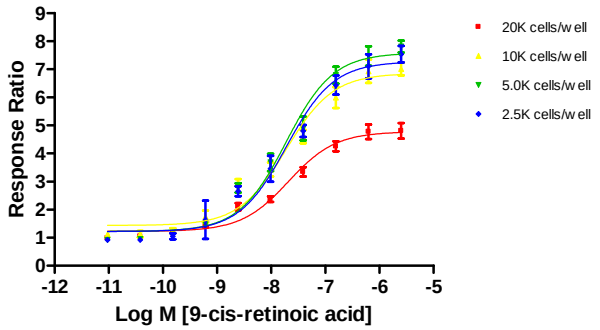
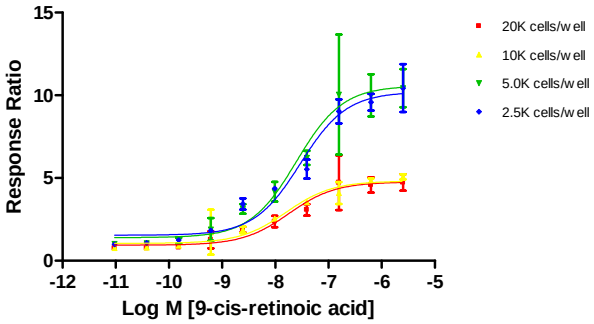


Figure 11c. – 9-cis-retinoic acid dose response with 20K, 10K, 5.0K, and 2.5K cells/well in a tissue culture treated plate.



RXR alpha-UAS-*bla* HEK 293T cells were plated the day of the assay at 20,000, 10,000, 5,000, or 2,500 cells/well in either a 384-well black-walled (a) biocoat, (b) Cellbind, or (c) tissue culture treated plate. Cells were stimulated with a serial dilution series of 9-cis-retinoic acid (Biomol cat# GR101) in the presence of 1.0% DMSO for 16 hours at 37ºC. Cells were loaded for 90 minutes with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM). Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the Response Ratios were plotted against the indicated concentrations of agonists (n=8 for each data point).

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