

GeneBLAzer® RXR α -UAS-*bla* HEK 293T Validated Assay

Cat. no. K1261

GeneBLAzer® Nuclear Hormone Receptor Cell-Based Assay Validation Packet

This cell-based assay has been thoroughly tested and validated by Invitrogen and is suitable for immediate use in a screening application. The following information illustrates the high level of assay testing completed and the validation of assay performance under optimized conditions.

Target Description

The Retinoid X receptor- α (RXR α) 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 NH₂-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 α belongs to the family of retinoid x receptors, one of two retinoic receptor families (retinoic acid receptors and retinoid x receptors). RXR α is one of three members of the RXR family which consists of RXR α , RXR β , and RXR γ (11). The A/B and D regions of RXR α 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 α agonist LG100268 activates the transcriptional response RXR:PPAR γ and RXR:LXR heterodimers alone and synergistically with PPAR γ and LXR agonists, but does not activate RXR:RAR and RXR:TR heterodimers whose activity is dependent upon RAR and TR agonists (10).

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

Cell Line Description

The Geneblazer RXR α -UAS-*bla* HEK 293T cells contain the ligand-binding domain (LBD) of the human retinoid X receptor- α fused to the DNA-binding domain of GAL4 stably integrated in the CellSensor UAS-*bla* HEK293T cell line. CellSensor UAS-*bla* HEK 293T cells (catalog#K1104) stably express a beta-lactamase reporter gene under the transcriptional control of a 7x Upstream Activator Sequence (UAS). Transcription from the 7xUAS is activated by the binding of the GAL4 transcription factor DNA-binding-domain (DBD). The GAL4-DBD is expressed as a fusion protein with the ligand binding domain (LBD) of RXR α . When an agonist binds to the LBD of the GAL4(DBD)-RXR α (LBD) fusion protein it translocates to the nucleus where it binds to the 7x UAS inducing transcription of beta-lactamase. RXR α -UAS-*bla* HEK 293T cells have been tested for assay performance using variable assay conditions, including DMSO concentration, cell number, stimulation time, substrate loading time and have been validated for Z' and EC₅₀ concentrations of 9-cis-retinoic acid. Additional testing data using alternate stimuli are also provided.

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=3)

9-cis-retinoic acid $EC_{50} = 5.7 \text{ nM}$
Z'-Factor (EC_{100}) = 0.71
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 $EC_{50} = 4.1 \text{ nM}$
Phytanic acid $EC_{50} = 4.3 \text{ }\mu\text{M}$
Docosahexaenoic acid $EC_{50} = >10 \text{ }\mu\text{M}$

3. Antagonist dose response

See antagonist dose response section

4. Cell culture and maintenance

See Cell Culture and Maintenance Section and Table 1

Assay Testing Summary

5. Assay performance with variable cell number

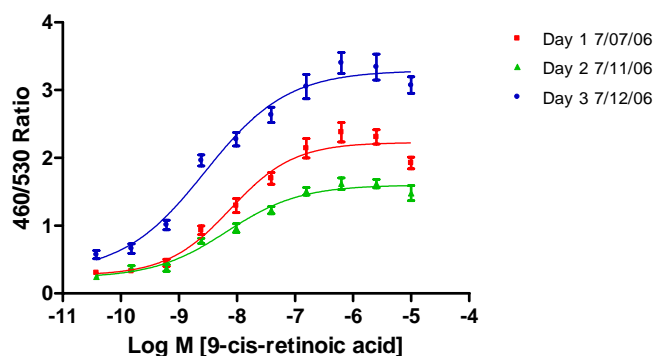
6. Assay performance with variable stimulation time

7. Assay performance with variable substrate loading time

8. Assay performance with variable DMSO concentration

Primary Agonist Dose Response

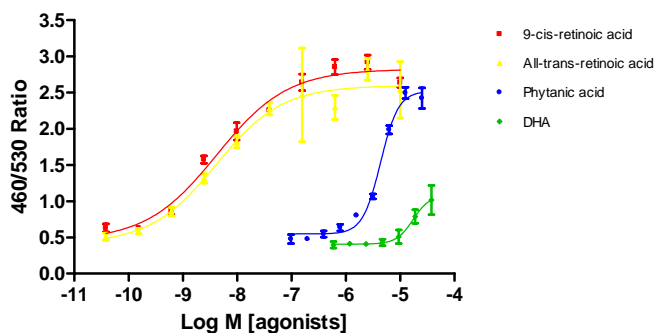
Figure 1 —9-cis-retinoic acid dose response under optimized conditions.



RXR α -UAS-*bla* HEK 293T cells were assayed on three separate days. Cells were plated the day of the assay in a 384-well format (10,000 cells/well) and stimulated with 9-cis-retinoic acid (Biomol #GR101) 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 (1 μM 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 each replicate plotted against the indicated concentrations of 9-cis-retinoic acid (n=16 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 α -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 (1 μM 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).

Antagonist Dose Response

There are currently no commercially available antagonists for RXR α .

Cell Culture and Maintenance

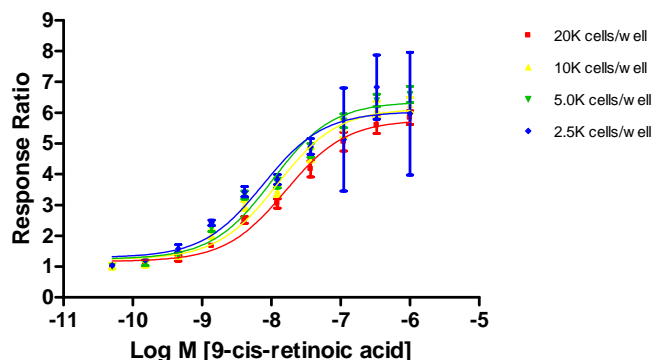
Cells should be maintained at between 5 and 90% confluency in complete growth media and in a humidified incubator at 37°C and 5% CO₂. Split cells at least twice a week. Do not allow cells to reach confluence.

Table 1 – 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

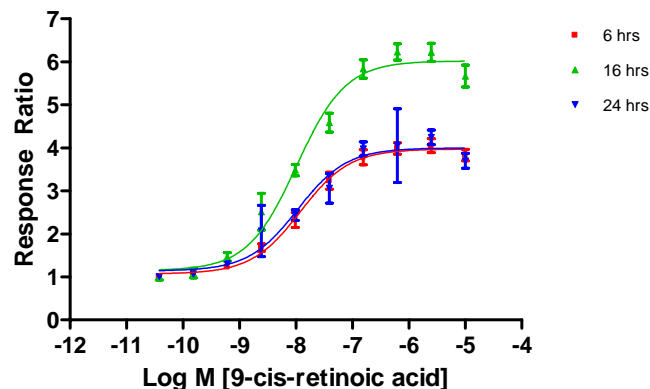
Figure 4— 9-cis-retinoic acid dose response with 2.5, 5.0, 10, and 20K cells/well



RXR α -UAS-*bla* HEK 293T cells were plated at 2500, 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 (1 μ M 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

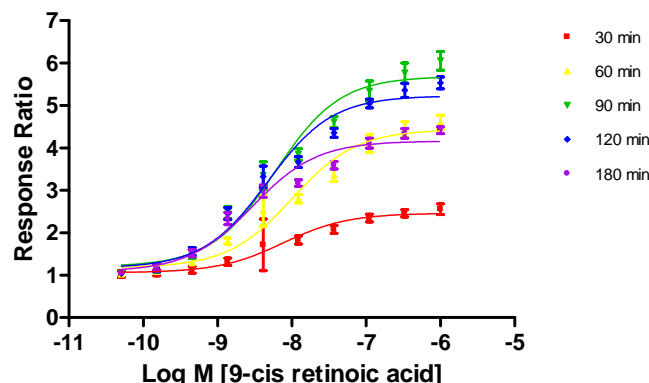
Figure 5 – Chenodeoxycholic acid dose response with 6, 16, and 24 hour stimulation times



RXR α -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 (1 μ M 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

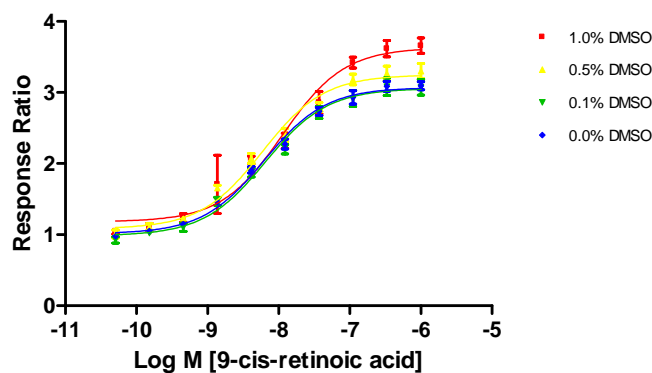
Figure 6 – 9-cis-retinoic acid dose response with 0.5, 1, 1.5, 2, and 3 hour loading times



RXR α -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 (1 μ M 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

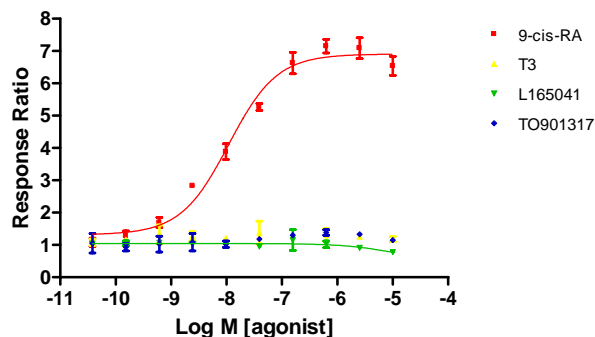
Figure 7 – 9-cis-Retinoic acid dose response with 0, 0.1, 0.5 and 1% DMSO.



RXR α -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 37°C. Cells were loaded for 90 minutes with LiveBLazer™-FRET B/G Substrate (1 μ M 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

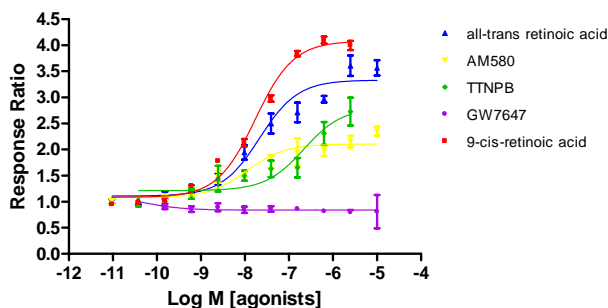
Figure 8 – Dose response of additional nuclear receptor agonists.



RXR α -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 37°C. Cells were loaded for 90 minutes with LiveBLazer™-FRET B/G Substrate (1 μ M 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

Figure 9 – Dose response of additional nuclear receptor agonists.



RXR α -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 37°C. Cells were loaded for 90 minutes with LiveBLazer™-FRET B/G Substrate (1 μ M 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 α antagonist RO41-5253

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

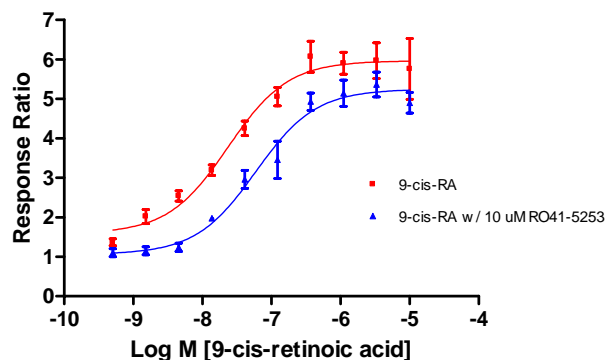


Figure 10b. – Dose response of all-trans-retinoic acid in the presence or absence of RO41-5253

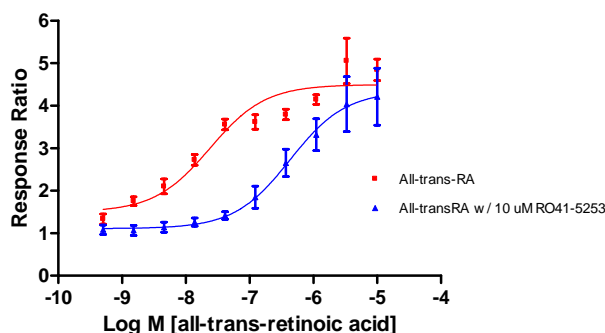


Figure 10c. – Dose response of RAR α agonist AM580 in the presence or absence of RO41-5253

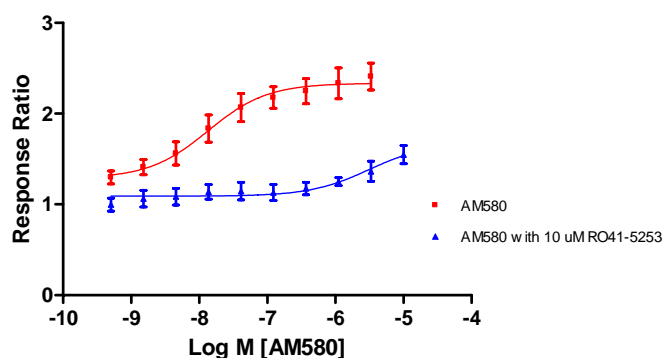
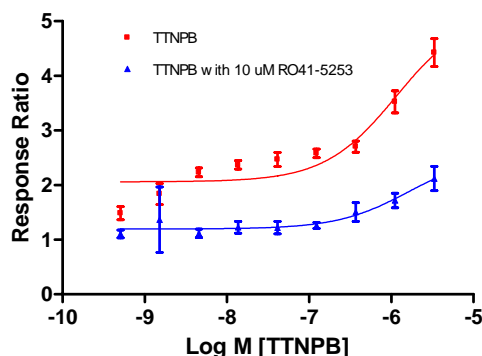


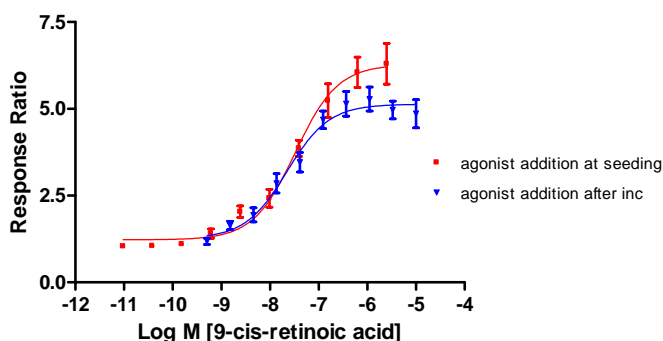
Figure 10d. – Dose response of TTNPB in the presence or absence of RO41-5253



RXR α -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 μ M 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 37°C. Cells were loaded for 90 minutes with LiveBLazer™-FRET B/G Substrate (1 μ M 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).

Addition of 9-cis-retinoic acid dose response upon seeding of the cells or after an incubation at 37°C for > 3 hours

Figure 11. Comparison of 9-cis-retinoic acid addition at time of cell seeding or after >3 hours at 37°C.



RXR α -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) added when cells were seeded or after an incubation of the cells at 37°C for >3hrs. Cells were stimulated with 9-cis-retinoic acid in the presence of 1.0% DMSO for 16 hours at 37°C. Cells were loaded for 90 minutes with LiveBLazer™-FRET B/G Substrate (1 μ M 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 12a. – 9-cis-retinoic acid dose response with 20K, 10K, 5.0K, and 2.5K cells/well in a poly-d-lysine (biocoat) plate.

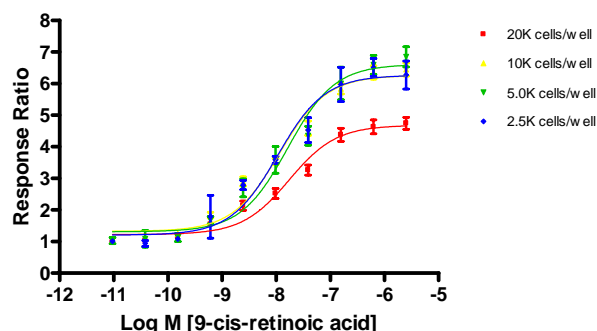


Figure 12b. – 9-cis-retinoic acid dose response with 20K, 10K, 5.0K, and 2.5K cells/well in a Cellbind plate.

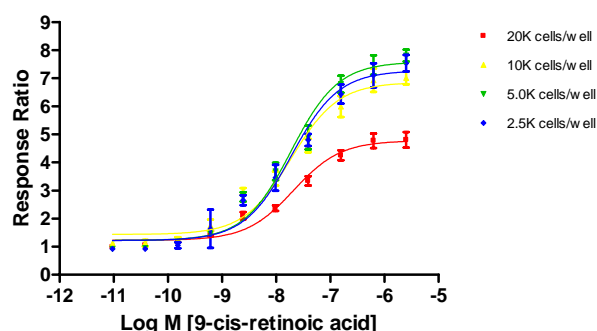


Figure 12c. – 9-cis-retinoic acid dose response with 20K, 10K, 5.0K, and 2.5K cells/well in a tissue culture treated plate.

RXR α -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 37°C. Cells were loaded for 90 minutes with LiveBLazer™-FRET B/G Substrate (1 μ M 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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