Validation & Assay Performance Summary

GeneBLAzer® RAR gamma DA Cells & Assay Kit

GeneBLAzer® RAR gamma-UAS-*bla* HEK 293T Cells

Cat. no. K1559, K1763

Target Description

Retinoic Acid Receptor gamma (RAR gamma) is a member of the Retinoic Acid Receptor family. Retinoids, vitamin A and its natural and synthetic analogues, are a very important group of hormones that regulate a wide variety of biological functions including embryogenesis, cell growth, and cell differentiation.[1] Retinoids have a wide variety of clinical benefit, they have uses in dermatology, oncology, diabetes, and diseases associated with HPV. Non-selective retinoids are usually associated with toxicity problems that limit their therapeutic usefulness. RAR gamma is the predominant RAR subtype present in the skin and it constitutes more than 90% of the total skin or keratinocyte RAR repertoire. [3] Skin irritation is a classical toxicity associated with RAR pan-agonists, like RA or TTNPB, and is believed to be a manifestation of RAR gamma activation. Furthermore, it has been shown that tumor-specific apoptosis can be driven by RAR gamma selective agonists, for pancreatic cancer cells. [6]

RAR beta and gamma are significantly different from RAR alpha in the way they bind to co-repressors. RAR beta and gamma have a fully functional SMRT docking site but the access of SMRT to this docking site is blocked by helix 12, which in RAR beta and gamma assumes a sequestered position in the absence of hormone. This ultimately leads to high basal levels of transcription even in the absence of ligand. This transcriptional activity can, however, be increased with the addition of the ligand all-trans retinoic acid (ATRA). [4,5]

Cell Line Description

GeneBLAzer® RAR gamma DA (Division Arrested) cells and RAR gamma-UAS-*bla* HEK 293T cells contain the ligand-binding domain (LBD) of the human retinoic acid receptor gamma (RAR gamma) 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)-RAR gamma (LBD) fusion protein, the protein binds to the UAS, resulting in expression of beta-lactamase.

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 RAR gamma DA cells and RAR gamma-UAS-*bla* HEK 293T cells are functionally validated for Z' and EC_{50} concentrations of *all-trans* retinoic acid (ATRA) (Figure 1). In addition, RAR gamma-UAS-*bla* HEK 293T cells have been tested for assay performance under variable conditions, including DMSO concentration, cell number, and stimulation time.

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)**

	DA	Dividing
All-trans retinoic acid EC_{50}	0.17 nM	0.19 nM
Z'-Factor (EC_{100})	0.92	0.89

Response Ratio = 20-30
Optimum cell no. = 5K cells/well
Optimum [DMSO] = up to 1%
Stimulation Time = 20-24 hours
Max. [Stimulation] = 10 nM

2. **Alternate agonist dose response**

TTNPB EC_{50} = 0.145 nM
CD437 EC_{50} = 2.67 nM
9-*cis*-retinoic acid EC_{50} = 3.0 nM
AM580 EC_{50} = 16.52 nM

3. **Antagonist dose response**

AGN 193109 IC_{50} = 0.1 nM

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

Assay Testing Summary

5. **Assay performance with variable cell number**

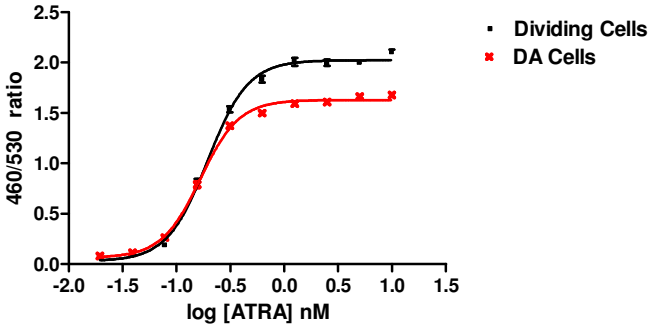
6. **Assay performance with variable stimulation time**

7. **Assay performance with variable substrate loading time**

8. **Assay performance with variable DMSO concentration**

Primary Agonist Dose Response

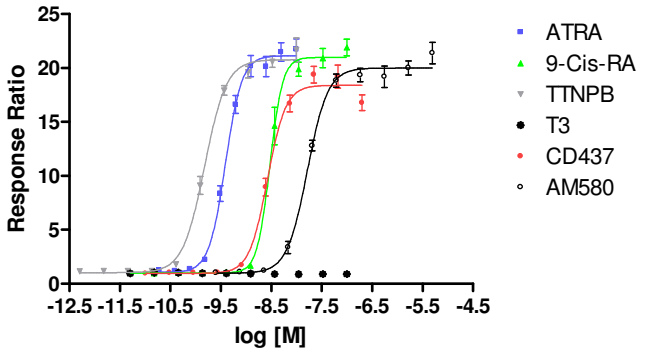
Figure 1 — RAR gamma DA and RAR gamma-UAS-*bla* HEK 293T dose response to *All-trans* retinoic acid under optimized conditions



RAR gamma DA cells and RAR gamma-UAS-*bla* HEK 293T cells were plated at 5,000 cells/well in a 384-well format and stimulated with *all-trans* retinoic acid (MP biomedicals #02190269) over the indicated concentration range in the presence of 0.5% DMSO for ~21 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 120 minutes. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader in bottom-read mode, and the background-corrected ratios were plotted against the indicated concentrations of *all-trans* retinoic acid (n= 16 for each data point).

Alternate Agonist Dose Response

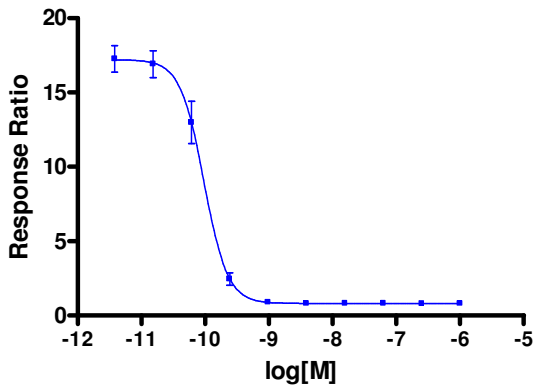
Figure 2 —Alternate agonist dose response



RAR gamma-UAS-*bla* HEK 293T cells were plated at 5,000 cells/well in a 384-well format. Cells were stimulated with either *all-trans* retinoic acid (MP biomedicals #02190269), 9-*cis*-retinoic acid (LKT labs #R1777), TTNPB (Tocris #0761), CD437 (Tocris #1549), AM580 (Tocris #0760), or T3 (CalBiochem #64245) over the indicated concentration range in the presence of 0.5% DMSO for 21 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 120 minutes. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader in bottom-read mode, and the background-corrected Response Ratios plotted against the indicated concentrations of the agonists (n= 8 or 16 for each data point).

Antagonist Dose Response

Figure 3 — AGN 193109 dose response



RAR gamma-UAS-*bla* HEK 293T cells (5,000 cells/well) were plated the day of the assay in a 384-well black-walled tissue culture assay plate in 0.5% DMSO. Cells were treated with AGN 193109 (refer to reference #7 and #8 for structure and synthesis) and incubated at 37 degrees C for 45 min., followed by 0.8nM *all-trans* retinoic acid agonist stimulation for 24 hours. Cells were then loaded for 2 hours with LiveBLAzer™-FRET B/G Substrate. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader in bottom-read mode, and the background-corrected Response Ratios plotted against the indicated concentrations of AGN 193109. The IC50 of AGN 193109 is ~0.1 nM (n=8 for each data point).

Table 1 – Dividing Cell Culture and Maintenance

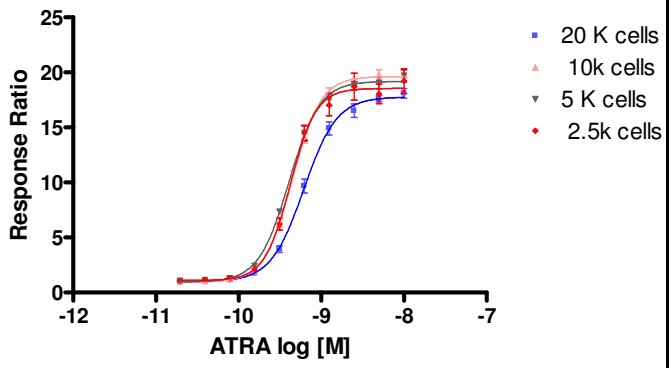
Cells should be maintained at between 10 and 95% confluency in complete growth media and in a humidified incubator at 37ºC and 5% CO_2. Split cells at least twice a week. Do not allow cells to reach confluence.

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%	
Na Pyruvate	—	—	1 mM	
NEAA	0.1 mM	0.1 mM	0.1 mM	—
HEPES (pH 7.3)	25 mM	25 mM	—	—
Hygromycin B	—	80 µg/mL	—	—
Zeocin™	—	80 µg/mL	—	—
Penicillin	100 U/mL	100 U/mL	100 U/mL	—
Streptomycin	100 µg/mL	100 µg/mL	100 µg/mL	—
Recovery™ Cell Culture Freezing Medium	—	—	—	100%

Assay Performance with Variable Cell Number

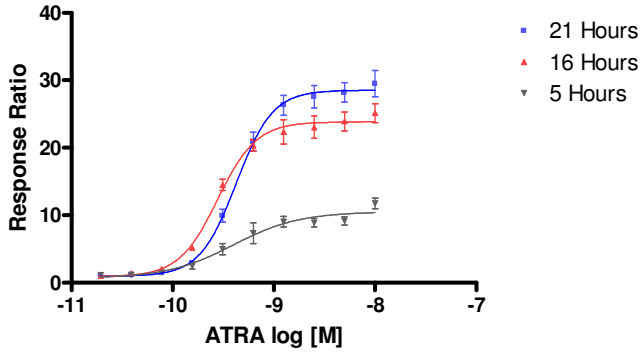
Figure 4— *All-trans* retinoic acid dose response with 2.5, 5, 10, and 20K cells/well



RAR gamma-UAS-*bla* HEK 293T cells were plated at 2500, 5000, 10,000, or 20,000 cells/well in a 384-well format. Cells were stimulated with all-trans retinoic acid (MP biomedicals #02190269) for ~21 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 120 minutes. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader in bottom-read mode, and the background-corrected Response Ratios plotted against the indicated concentrations of *all-trans* retinoic acid (n=8 for each data point).

Assay performance with Variable Stimulation Time

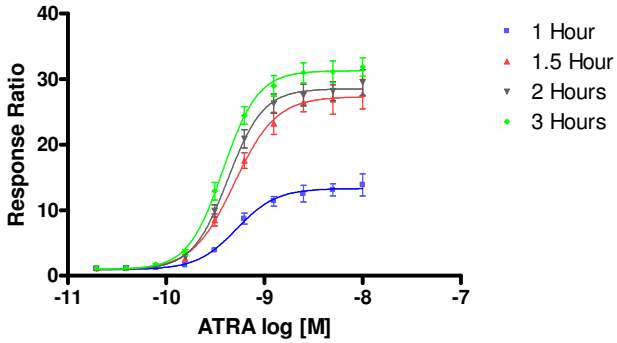
Figure 5 – *All-trans* retinoic acid dose response with 5, 16, and 21 hour stimulation times



RAR gamma-UAS-*bla* HEK 293T cells were plated at 5,000 cells/well in a 384-well format the day of the assay. *All-trans* retinoic acid (MP biomedicals #02190269) was then added to the plate over the indicated concentration range for 5, 16, and 21 hours in the presence of 0.5% DMSO and then loaded for 120 minutes with LiveBLAzer™-FRET B/G Substrate. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader in bottom-read mode, and the background-corrected Response Ratios plotted against the indicated concentrations of *all-trans* retinoic acid (n=16 for each data point).

Assay performance with Variable Substrate Loading Time

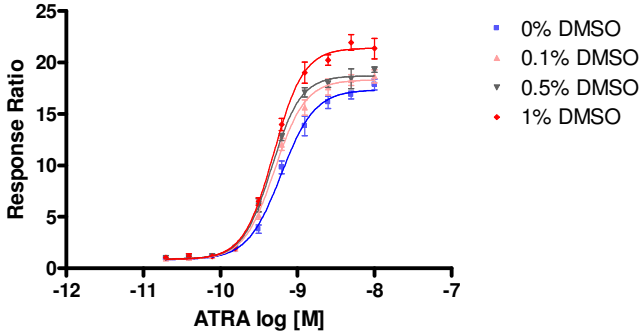
Figure 6 – *All-trans* retinoic acid dose response with 1, 1.5, 2, and 3 hour loading times



RAR gamma-UAS-*bla* HEK 293T cells were plated at 5,000 cells/well in a 384-well format the day of the assay. Cells were stimulated with all-trans retinoic acid (MP biomedicals #02190269) in the presence of 0.5% DMSO for ~21 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for either 1, 1.5, 2, or 3 hours. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader in bottom-read mode, and the background-corrected Response Ratios plotted against the indicated concentrations of *all-trans* retinoic acid (n=16 for each data point).

Assay Performance with variable DMSO concentration

Figure 7 – *All-trans* retinoic acid dose response with 0, 0.1, 0.5 and 1% DMSO.



RAR gamma-UAS-*bla* HEK 293T cells were plated at 5,000 cells/well in a 384-well format the day of the assay. *All-trans* retinoic acid (MP biomedicals #02190269) was then added to the plate over the indicated concentration range. DMSO was added to the assay at concentrations from 0% to 1%. Cells were stimulated for ~21 hrs with agonist and loaded for 120 minutes with LiveBLAzer™-FRET B/G Substrate. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader in bottom-read mode, and the background-corrected Response Ratios plotted against the indicated concentrations of *all-trans* retinoic acid (n=8 for each data point).

References

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