Validation & Assay Performance Summary

GeneBLAzer® RAR beta DA Cells & Assay Kit

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

Cat. no. K1407, K1695

Target Description

RAR beta is a member of the Retinoic Acid Receptor family of nuclear receptors. 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. Currently, research is being done to find more receptor-selective retinoids, as well as function-selective retinoids, such as inverse agonists and antagonists.[3] RAR beta is also viewed as a tumor suppressor. It is often lost or down-regulated in breast cancer and breast cancer cell lines. It is often silenced during cancer progression, and re-expression can restore RA-mediated growth control.[6] Cotransfection experiments showed that RARs are activated by either all-trans- or 9-*cis*-retinoic acid at a ligand concentration of 5 X 10^{-8} mmol/L.[2]

RAR beta and gamma are significantly different from RAR alpha in the way they bind to co-repressors. RAR alpha binds co-repressor SMRT in the absence of ligand, but RAR beta and gamma interact only very weakly with this co-repressor, due to the conformation of helix 12 in these two receptors. 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 beta DA (Division Arrested) cells and RAR beta-UAS-*bla* HEK 293T cells contain the ligand-binding domain (LBD) of the human retinoic acid receptor beta (RAR beta) 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 beta (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 beta DA cells and RAR beta-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 beta-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}	0.58 nM	0.77 nM
Z'-Factor (EC_{100})	0.74	0.87

Response Ratio	= 3-5
Optimum cell no.	= 10K cells/well
Optimum [DMSO]	= up to 1%
Stimulation Time	= 18 hours
Max. [Stimulation]	= 10 nM

2. **Alternate agonist dose response**

TTNPB EC_{50}	= 0.09 nM
Ch55 EC_{50}	= 0.12 nM
9-cis-retinoic acid EC_{50}	= 0.3 nM
AM580 EC_{50}	= 2.9 nM
Fenretinide EC_{50}	= 220 nM

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**

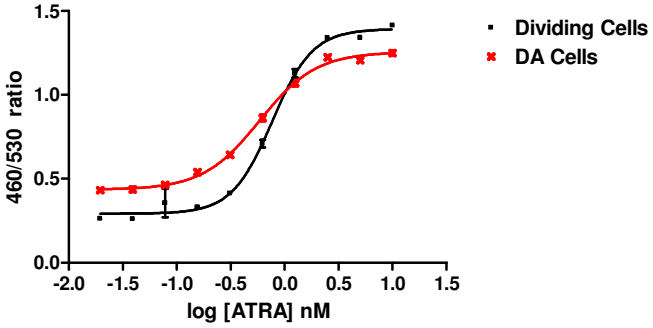
6. **Assay performance with variable stimulation time**

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

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

Primary Agonist Dose Response

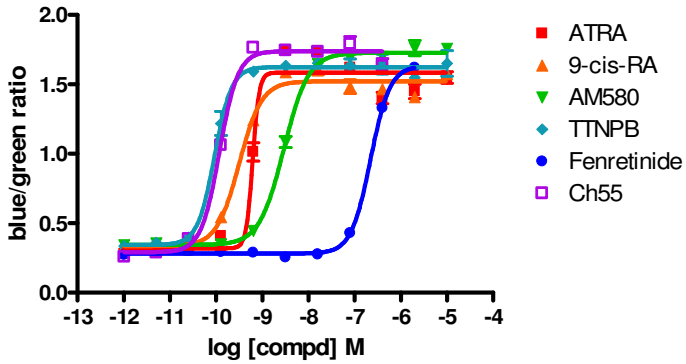
Figure 1 — RAR beta DA and RAR beta-UAS-*bla* HEK 293T dose response to *All-trans* retinoic acid under optimized conditions



RAR beta DA cells and RAR beta-UAS-*bla* HEK 293T cells were plated at 10,000 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 18 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 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

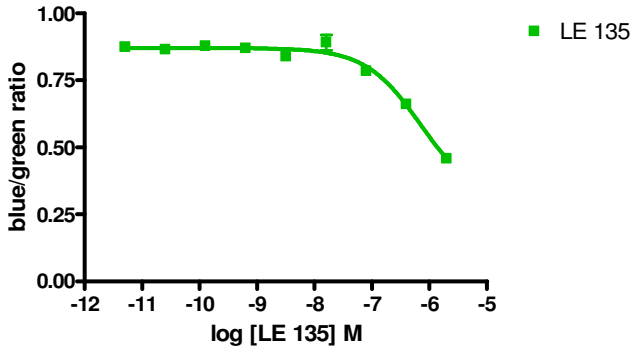
Figure 2 —Alternate agonist dose response



RAR beta-UAS-*bla* HEK 293T cells were starved 24 hours prior to assay and then plated at 10,000 cells/well in a 384-well format. Cells were stimulated with either *all-trans* retinoic acid (ATRA) (MP biomedicals #02190269), *9-cis*-retinoic acid (LKT labs #R1777), AM580 (Tocris #0760), TTNPB (Tocris #0761), Fenretinide (Tocris # 1396), or Ch55 (Tocris #2020) over the indicated concentration range in the presence of 0.5% DMSO for 18 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 in bottom-read mode, and the background-corrected ratios were plotted against the indicated concentrations of the agonists (n= 4 for each data point).

Antagonist Dose Response

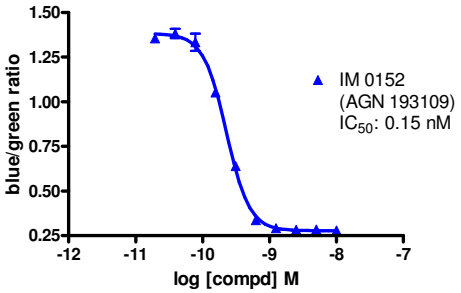
Figure 3 — LE 135 dose response



RAR beta-UAS-*bla* HEK 293T cells were starved for 24 hours prior to assay, and then plated at 10,000 cells/well in a 384-well black-walled tissue culture assay plate. Cells were treated with LE 135 (Tocris #2021) and incubated at 37 degrees C for 30 min., followed by 1.5 nM all-trans retinoic acid agonist stimulation for 18 hours. Cells were then loaded for 90 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 ratios are shown plotted against the indicated concentrations of LE 135. High concentrations of LE 135 are toxic to the cells so the inhibition curve cannot be completed. (n= 4 for each data point).

Antagonist Dose Response continued

Figure 4 — AGN 193109 dose response



RAR beta-UAS-*bla* HEK 293T cells were starved for 24 hours prior to assay, and then plated at 10,000 cells/well in a 384-well black-walled tissue culture assay plate. Cells were treated with AGN 193109 (see references 7 and 8 for structure) and incubated at 37 degrees C for 30 min., followed by 1 nM all-trans retinoic acid agonist stimulation for 18 hours. Cells were then loaded for 90 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 ratios are shown plotted against the indicated concentrations of AGN 193109. The IC50 of AGN 193109 is ~0.15 nM (n= 8 for each data point).

Dividing Cell Culture and Maintenance

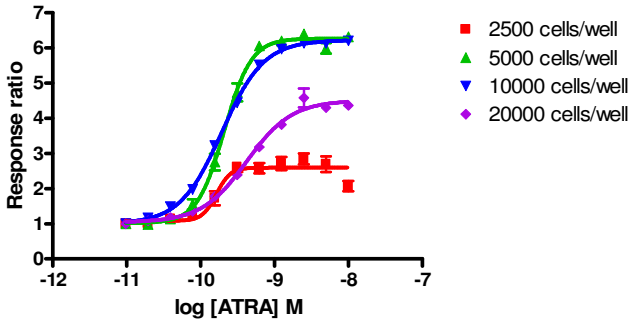
Cells should be maintained between 5 and 95% confluency in complete growth media in a humidified incubator at 37ºC and 5% CO_2. Split cells at least twice a week. Do not allow cells to reach confluence.

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	—	—	99.99%	—
Dialyzed FBS **Do not substitute!**	10%	10%	—	—
BSA	—	—	0.01%	
NEAA	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	—	—
Streptomycin	100 µg/mL	100 µg/mL	—	—
Recovery™ Cell Culture Freezing Medium	—	—	—	100%

Assay Performance with Variable Cell Number

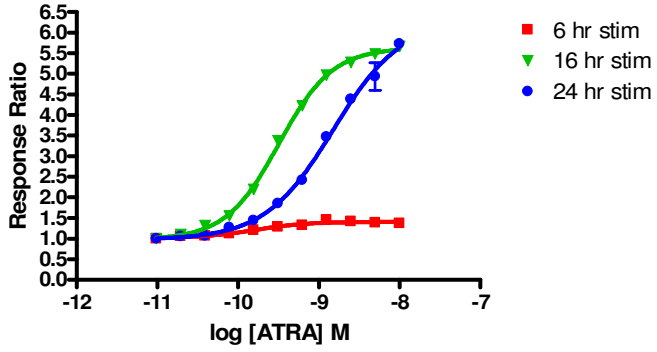
Figure 5— *All-trans* retinoic acid dose response with 2.5, 5, 10, and 20K cells/well



RAR beta-UAS-*bla* HEK 293T cells were starved for 24 hours prior to assay and then 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 18 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 for the various cell numbers were obtained using a standard fluorescence plate reader in bottom-read mode, and the background-corrected Response Ratios were plotted against the indicated concentrations of all-trans retinoic acid (n=8 for each data point).

Assay performance with Variable Stimulation Time

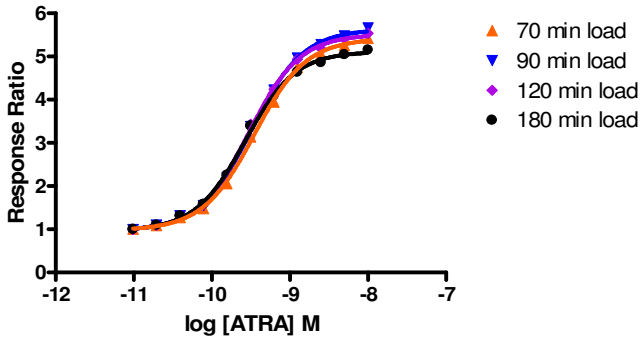
Figure 6 – *All-trans* retinoic acid dose response with 6, 16, and 24 hour stimulation times



RAR beta-UAS-*bla* HEK 293T cells were starved for 24 hours prior to assay and then plated at 10,000 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 6, 16, and 24 hours in 0.5% DMSO and then loaded for 90 minutes with LiveBLAzer™-FRET B/G Substrate(1 µM final concentration of CCF4-AM). 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 were plotted (n=8 for each data point).

Assay performance with Variable Substrate Loading Time

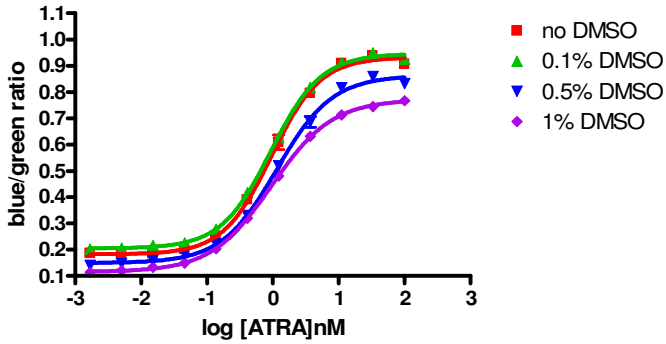
Figure 7 – *All-trans* retinoic acid dose response with 1, 1.5, 2, and 3 hour loading times



RAR beta-UAS-*bla* HEK 293T cells were starved for 24 hours prior to assay and then plated at 10,000 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 16 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate (1 µM final concentration of CCF4-AM) for either 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 in bottom-read mode, and the background-corrected Response Ratios were plotted against the indicated concentrations of all-trans retinoic acid (n=8 for each data point).

Assay Performance with variable DMSO concentration

Figure 8 – *All-trans* retinoic acid dose response with 0, 0.1, 0.5 and 1% DMSO.



RAR beta-UAS-*bla* HEK 293T cells were starved for 24 hours prior to assay and then plated at 15,000 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 18 hrs with agonist and loaded for 90 minutes with LiveBLAzer™-FRET B/G Substrate (1 µM final concentration of CCF4-AM). 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 are shown plotted for each DMSO concentration against the indicated concentrations of *all-trans* retinoic acid (n=8 for each data point).

References

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