Validation & Assay Performance Summary

GeneBLAzer® RAR alpha DA Assay Kit

GeneBLAzer® RAR alpha-UAS-*bla* GripTite™ Cells

Cat. no. K1387, K1685

Target Description

The retinoic acid receptor alpha (RAR 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). The RAR DNA binding domain recognizes and interacts with the retinoic acid receptor response elements (RAREs) (2). RAR binds as a heterodimer with RXR. The RAR/RXR heterodimer is associated with corepressor proteins at the RAREs in the absence of ligand. Upon binding of ligand, RAR undergoes a conformational change, releasing corepressor proteins and allowing for the interaction with coactivator proteins that enhance RARE driven gene transcription (2).

RAR alpha 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 (3). 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 (4).

Cell Line Description

The GeneBLAzer® RAR alpha DA (Division Arrested) and RAR alpha-UAS-*bla* HEK 293T cells contain the ligand-binding domain (LBD) of the human retinoic acid receptor alpha fused to the DNA-binding domain of GAL4 stably integrated in the GeneBLAzer® UAS-*bla* HEK293T cell line. GeneBLAzer® 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 RAR alpha. When an agonist binds to the LBD of the GAL4(DBD)-RAR alpha(LBD) fusion protein it translocates to the nucleus where it binds to the 7x UAS inducing transcription of beta-lactamase. Division Arrested (DA) cells are available in an Assay Kit (which includes cells and sufficient substrate to analyze 1 x 384-well plate).

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 alpha DA cells and RAR alpha-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 EC_{50} concentrations of all-trans retinoic acid. 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=3)**

   ATRA EC_{50} = 0.20-0.34nM
   Z'-Factor (EC_{100}) = 0.9
   Response Ratio = 21.9-22.3

   Optimum cell no. = 10K cells/well
   Optimum [DMSO] = up to 0.5%
   Stimulation Time = 16-24 hours
   Max. [Stimulation] = 100 nM

2. **Alternate agonist dose response**

   9-cis-RA EC_{50} = 0.83 nM
   AM580 EC_{50} = 0.17 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**

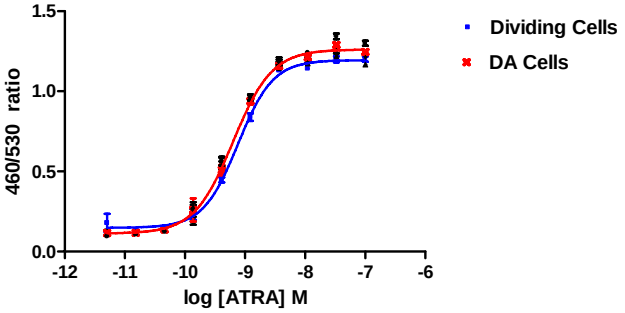
6. **Assay performance with variable stimulation time**

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

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

Primary Agonist Dose Response

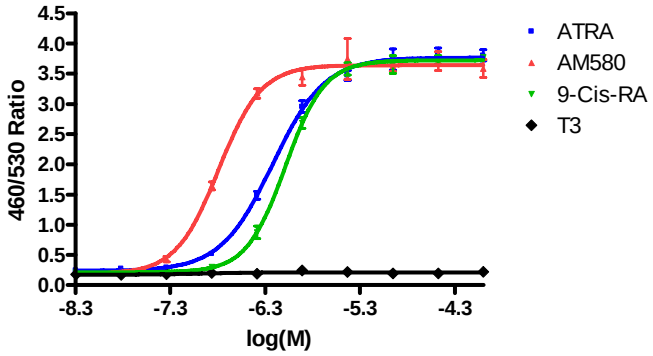
Figure 1 — RAR alpha-UAS-*bla* HEK293T dose response to known agonist ATRA



RAR alpha DA cells and RAR alpha-UAS-*bla* HEK 293T cells (10,000 cells/well) were assayed on three separate days. Cells were serum starved for 24 hrs then plated the day of the assay in a 384-well format and stimulated with ATRA (Sigma #R2625) over the indicated concentration range 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 120 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 ATRA (n= 16 for each data point).

Alternate Agonist Dose Response

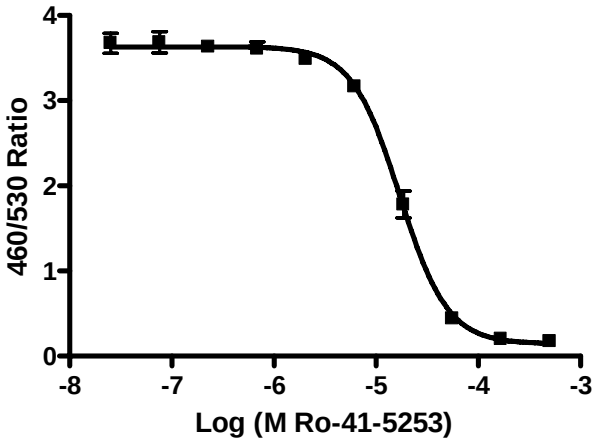
Figure 2 —ATRA, AM580, 9-cis-RA and T3(thyroid receptor hormone) Dose Response



RAR alpha-UAS-*bla* HEK 293T cells were starved for 24hrs, then (10,000 cells/well) were plated the day of the assay in a 384-well format. Cells were stimulated with either ATRA (Sigma #R2625), AM580 (Sigma # H8843), 9-cis-RA (BIOMOL #GR101-0025), or T3 (Calbiochem #642511) over the indicated concentration range 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 120 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

Figure 3 — RAR antagonist Ro-41-5253



RAR alpha-UAS-*bla* HEK 293T cells were serum starved for 24 hours, then (10,000 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 RAR antagonist Ro-41-5253 (BIOMOL # GR110) and incubated at 37 degrees C for 30 min., followed by 1nM ATRA agonist stimulation for 16 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 and the Ratios are shown plotted against the indicated concentrations of Ro-41-5253.

Dividing Cell Culture and Maintenance

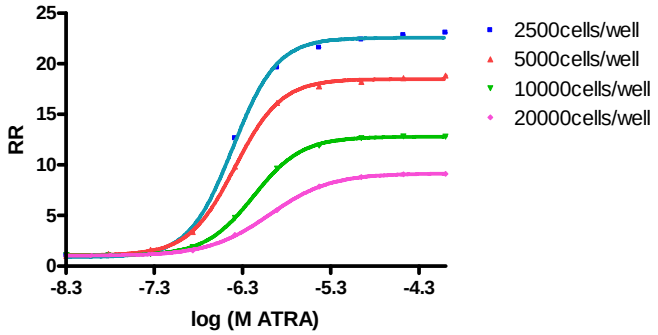
Cells should be maintained at between 5 and 90% confluency in complete growth media and in a humidified incubator at 37ºC and 5% CO_2. Split cells at least twice a week. Do not allow cells to reach confluence.

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	—	80 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

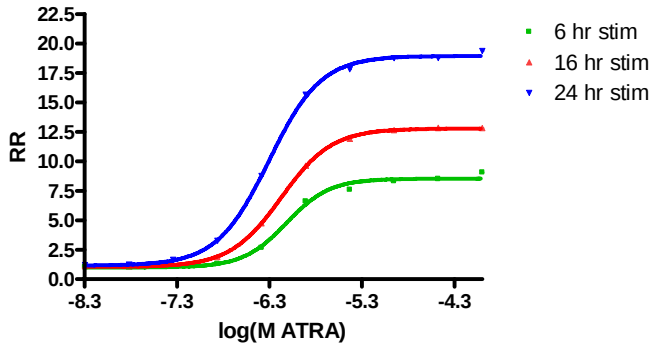
Figure 4— ATRA dose response with 2.5, 5, 10, and 20K cells/well



RAR alpha-UAS-*bla* HEK 293T cells were serum starved for 24 hrs, then plated at 2500, 5000, 10,000, or 20,000 or cells/well in a 384-well format the day of the assay in 0.5%DMSO. Cells were stimulated with ATRA (Sigma #R2625) 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 for the various cell numbers were obtained using a standard fluorescence plate reader and the Response Ratios (RR) plotted against the indicated concentrations of ATRA (n=8 for each data point).

Assay performance with Variable Stimulation Time

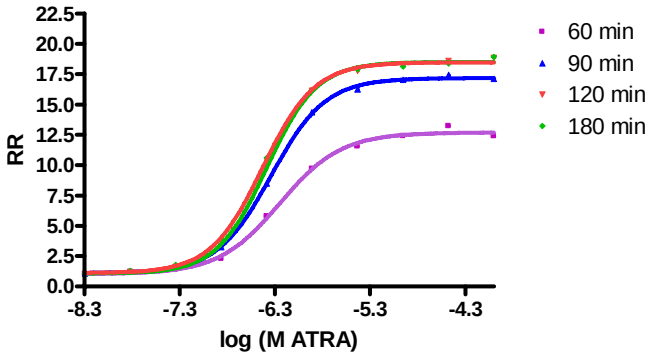
Figure 5 – ATRA dose response with 6, 16, and 24 hour stimulation times



RAR alpha-UAS-*bla* HEK 293T cells (10,000cells/well) were serum starved for 24 hrs, then plated the day of the assay in a 384-well black-walled tissue culture assay plate in 0.5% DMSO. ATRA (Sigma #R2625) was then added to the plate over the indicated concentration range for 6, 16, and 24 hours and then loaded for 2 hours 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 (RR) plotted against the indicated concentrations of ATRA (n=8 for each data point)

Assay performance with Variable Substrate Loading Time

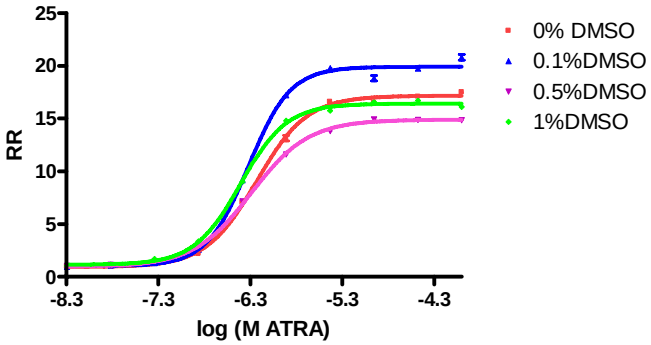
Figure 6 – ATRA dose response with , 60, 90, 120, and 180 minutes loading times



RAR alpha-UAS-*bla* HEK 293T cells were serum starved for 24 hrs, then plated at 10,000 cells/well in a 384-well format the day of the assay in 0.5%DMSO. Cells were stimulated with ATRA (Sigma #R2625) for 16 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for either 60, 90, 120, or 180 minutes. 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 (RR) plotted against the indicated concentrations of ATRA (n=8 for each data point).

Assay Performance with variable DMSO concentration

Figure 7 – ATRA dose response with 0, 0.1, 0.5 and 1% DMSO.



RAR alpha-UAS-*bla* HEK 293T cells (10,000 cells/well) were serum starved fot 24 hrs, then plated the day of the assay in a 384-well black-walled tissue culture assay plate. ATRA (Sigma #R2625) 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 16 hrs with agonist and loaded for 2 hours 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 (RR) are shown plotted for each DMSO concentration against the indicated concentrations of ATRA (n=8 for each data point).

References

1. Maurizio, C., Nico, M., De Fabiani, E. (2004) **Lipid-activated nuclear receptors: from gene transcription to the control of cellular metabolism**. *Eur. J. Lipid Sci. Technol*., **106**, 432-450.

2. Lamour F, Lardelli, P, Apfel, C. (1996) **Analysis of the ligand-binding domain of human RARalpha by site-directed mutagenesis**. *Mol. & Cell Biol.* **16**, 5386-5393.

3. Sussman F, de Lera AR (2005).**Ligand recognition by RAR and RXR receptors: binding and selectivity.***J. Med. Chem.* ***48***, 6212-6219.

4. Kizaki M, Ikeda Y, Tanosaki R, Nakajima H, Morikawa M, Sakashita A, Koeffler (1993) **HP.Effects of novel retinoic acid compound, 9-cis-retinoic acid, on proliferation, differentiation, and expression of retinoic acid receptor-alpha and retinoid X receptor-alpha RNA by HL-60 cells**. *Blood.* ***, 82***, 3592-3599