Validation & Assay Performance Summary

GeneBLAzer® PPAR delta DA Assay Kit

GeneBLAzer® PPAR delta DA Cells

GeneBLAzer® PPAR delta-UAS-*bla* HEK 293T Cells

Cat. no. K1395, K1690

Target Description

Peroxisome proliferators-activated receptors (PPARs) are ligand inducible transcription factors of the nuclear receptor superfamily, capable of acting as co-repressors and/or co-activators 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) [4].

Three distinct subtypes of PPARs include PPAR alpha, PPAR delta and PPAR gamma, respectively [1-3]. All PPAR subfamily members heterodimerize with the receptor for 9-*cis* retinoic acid (RXR) [7] and bind to target gene peroxisome proliferators elements (PPREs), a direct repeat of the sequence AGGTCA separated by one nucleotide (DR-1) [2]. Although the function of PPAR delta, which is expressed ubiquitously, is less well known, this subtype may be involved in the regulation of cholesterol and lipid metabolism [5-6], in keratinocyte proliferation [8] and in apoptosis [9]. Among the three PPARs, PPAR delta distinguished itself by displaying remarkably potent transcriptional repression activity. Agonist binding to PPAR delta ligand binding domain provokes a conformational change that produces a suitable binding surface for recruitment of coactivators [10]. These conformational changes lead to a decrease in the affinity of transcription co-repressors and the interaction with transcription co-activators. These co-activators and co-repressors regulate gene transcription by interacting with the transcriptional pre-initiation complex and histone acetyl transferases [4].

Cell Line Description

GeneBLAzer® PPAR delta DA (Division Arrested) cells and PPAR delta-UAS-*bla* HEK 293T cells contain the ligand-binding domain (LBD) of the human Peroxisome Proliferator-Activated Receptor-delta (PPAR delta) 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)-PPAR delta (LBD) fusion protein, the protein binds to the UAS, resulting in expression of beta-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 PPAR delta DA cells and PPAR delta-UAS-*bla* HEK 293T cells are functionally validated for Z' and EC_{50} concentrations of L-165041 (Figure 1). In addition, PPAR delta-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
L-165,041 EC_{50}	40nM	26nM
Z'-Factor (EC_{100})	0.94	0.78

Response Ratio	= 15
Optimum cell no.	= 10K cells/well
Optimum [DMSO]	= up to 1%
Optimum Stim Time	= 24 hours
Max. [Stimulation]	= 1 µM

2. **Alternate agonist dose response**

GW0742 EC_{50}	= 1.7 nM
GW7647 EC_{50}	= 190 nM
Retinoic acid EC_{50}	= 48 nM
Bezafibrate EC_{50}	= 102 µM

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**

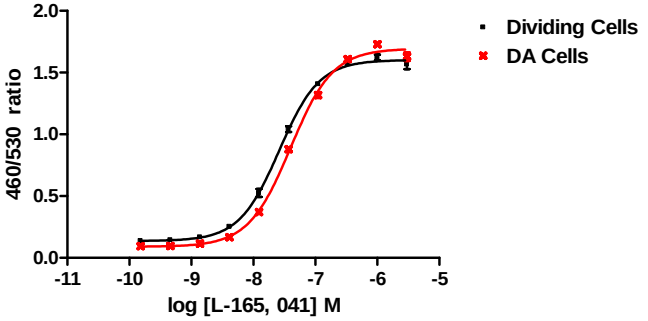
6. **Assay performance with variable stimulation time**

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

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

Primary Agonist Dose Response

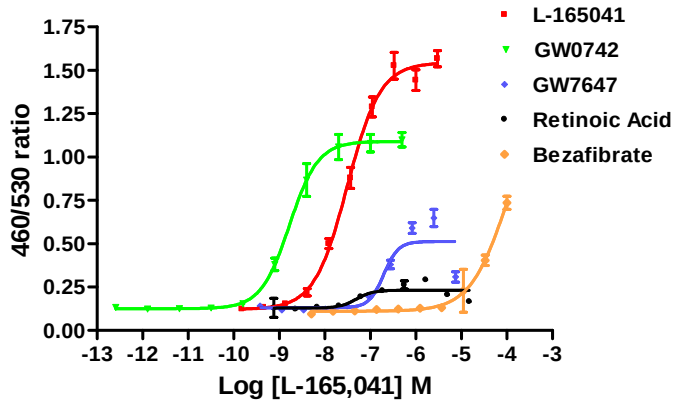
Figure 1 — PPAR delta DA and PPAR delta-UAS-*bla* HEK 293T dose response to L-165,041 under optimized conditions



PPAR delta DA cells and PPAR delta-UAS-*bla* HEK 293T cells (10,000 cells/well) were plated in a 384-well format stimulated with a dilution series of L-165,041 in the presence of 0.5% DMSO for 24 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 L-165,041 (n=6 for each data point).

Alternate Agonist Dose Response

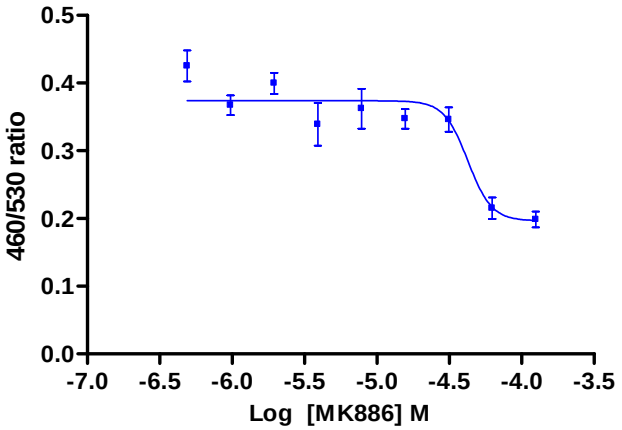
Figure 2 — PPAR delta-UAS-*bla* HEK 293T dose response to L-165041, GW0742, GW7647, Retinoic acid and Bezafibrate



PPAR delta-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 L-165,041 (Sigma #L2167), GW0742 (Sigma #G3295), GW7647 (CalBiochem#370698), Retinoic acid (BioMol #GR101-0005), and Bezafibrate (Sigma #B7273) over the indicated concentration range in the presence of 0.5% DMSO for 24 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 — PPAR delta-UAS-*bla* HEK 293T dose response to MK886



PPAR delta-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. Cells were treated with MK886 (Sigma #M2692) and incubated at 37 degrees C for 45 min., followed by 25 nM L-165,041 agonist stimulation for 24 hours in 0.5% DMSO. Cells were 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 and the Ratios are shown plotted against the indicated concentrations of L-165,041.

Dividing Cell Culture and Maintenance

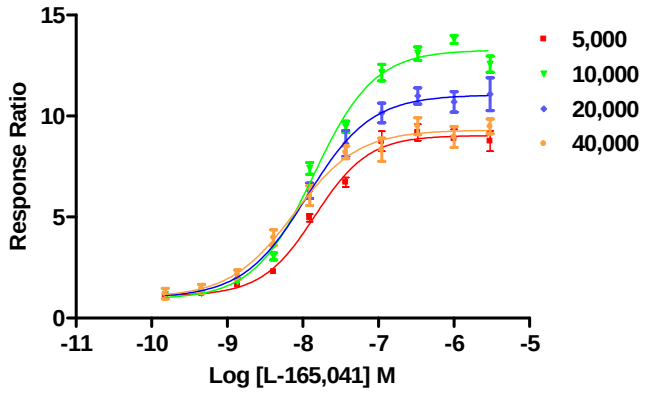
Dividing cells should be maintained at between 5 and 90% confluency in complete growth media and in a humidified incubator at 37ºC and 5% CO_2. Split dividing cells at least twice a week. Do not allow dividing cells to reach confluence.

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	—	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

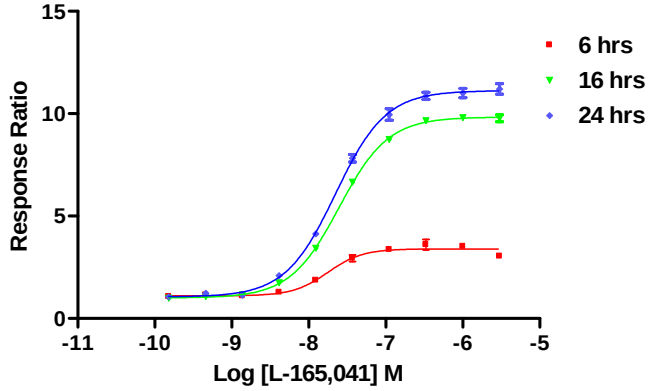
Figure 4— PPAR delta-UAS-*bla* HEK 293T dose response to L-165,041 with 5, 10, 20, and 40K cells/well



PPAR delta-UAS-*bla* HEK 293T cells were plated at 5000, 10,000, 20,000 or 40,000 cells/well in a 384-well format the day of the assay. Cells were stimulated with L-165,041 (Sigma #L2167) for 24 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 plotted against the indicated concentrations of L-165,041 (n=8 for each data point).

Assay performance with Variable Stimulation Time

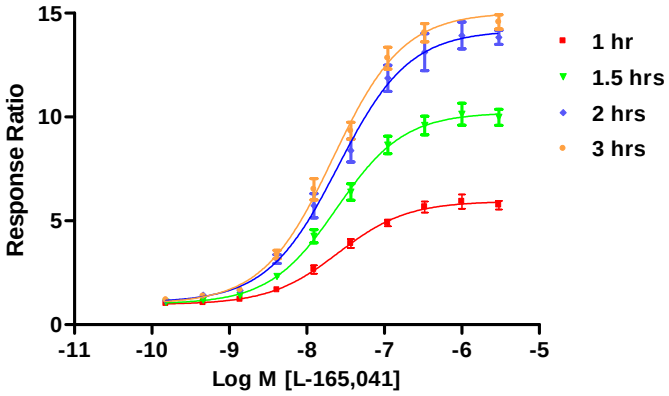
Figure 5 – PPAR delta-UAS-*bla* HEK 293T dose response to L-165,041 with 6, 16, and 24 hour stimulation times



PPAR delta-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. L-165,041 (Sigma #L2167) 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 120 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 for each stimulation time plotted against the indicated concentrations of L-165,041 (n=8 for each data point).

Assay performance with Variable Substrate Loading Time

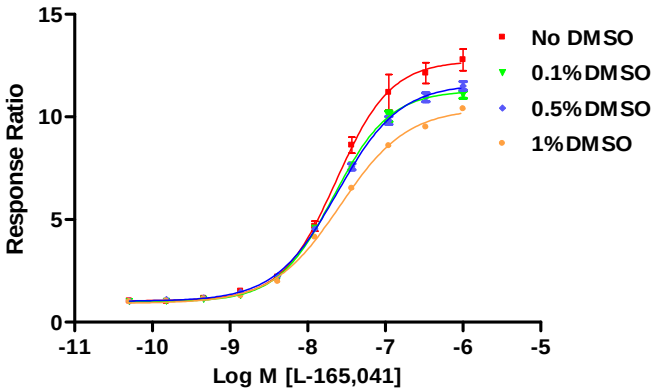
Figure 6 – PPAR delta-UAS-*bla* HEK 293T dose response to L-165,041 with 1, 1.5, 2, and 3 hour loading times



PPAR delta-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 L-165,041 (Sigma # L2167) in the presence of 0.5% DMSO for 24 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for either 1, 1.5, 2, and 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 for each substrate loading time plotted against the indicated concentrations of L-165,041 (n=8 for each data point).

Assay Performance with variable DMSO concentration

Figure 7 – PPAR delta-UAS-*bla* HEK 293T dose response to L-165,041 with 0, 0.1, 0.5 and 1% DMSO.



PPAR delta-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. L-165,041 (Sigma # L2167) 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 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 for each DMSO concentration plotted against the indicated concentrations of L-165,041 (n=8 for each data point).

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