Validation & Assay Performance Summary

GeneBLAzer® PPAR alpha UAS-*bla* HEK 293T Validated Assay

Cat. no. K1875

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 peroxisome proliferator-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 are known as PPAR alpha, PPAR beta/delta, and PPAR gamma respectively [1-3]. All of 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]. PPAR alpha is expressed mainly in the liver, heart, kidneys, and brain [5]. It plays a role in the uptake and oxidation of fatty acids and lipoprotein metabolism. Activation of PPAR alpha by fibrates lowers triglycerides, raises HDL, and has insulin sensitizing effects [6]. PPAR alpha also regulates neural cell differentiation and has been associated with neurodegeneration and inflammation [5]. Fatty acids, hypolipidemic drugs, and xenobiotics are all ligands of PPAR alpha [8].

Cell Line Description

The GeneBLAzer® PPAR alpha UAS-*bla* HEK 293T cells contain the ligand-binding domain (LBD) of the human PPAR alpha (accession #NM_005036) fused to the DNA-binding domain of GAL4 transiently transduced via BacMam virus into 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 PPAR alpha. When an agonist binds to the LBD of the GAL4(DBD)-PPAR alpha (LBD) fusion protein, the GAL4 DBD binds to the 7x UAS inducing transcription of beta-lactamase. PPAR alpha UAS-*bla* HEK 293T cells have been tested for assay performance using variable assay conditions, including cell number, stimulation time, substrate loading time and have been validated for Z' and EC_{50} concentrations of GW7647. Additional testing data using alternate stimuli are also provided. While this assay has a lower assay window and Z' than other GeneBLAzer assays due to the biology of the receptor, it is still suitable for profiling. This assay provides accurate pharmacology and provides robust EC_{50} and IC_{50} values.

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)**

   GW7647 EC_{50} = 0.14 nM
   Z'-Factor (EC_{100}) = 0.58
   Response Ratio = 2.7

   Optimum cell no. = 20K cells/well
   Stimulation Time = 16-18 hours
   Max. [Stimulation] = 50 nM

2. **Alternate ligand dose response**

   GW9662 EC_{50} = 0.52 nM
   GW0742 EC_{50} = 68 nM
   GW501516 EC_{50} = 92 nM
   L165,041 EC_{50} = 164 nM
   rosiglitazone EC_{50} = 780 nM
   GW1929 EC_{50} = 1750 nM

   Inverse agonist/antagonist:
   GW6471 EC_{50} = 160 nM

Assay Testing Summary

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

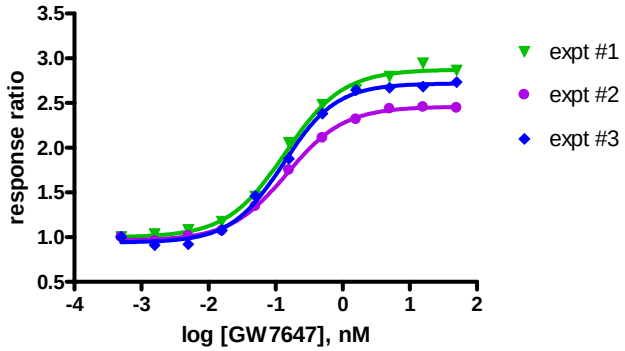
4. **Assay performance with variable stimulation time**

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

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

Primary Agonist Dose Response

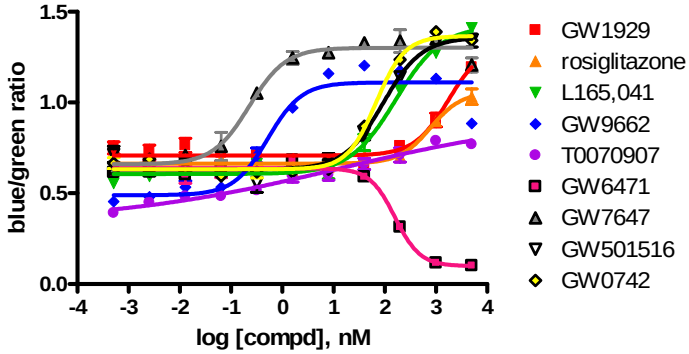
Figure 1 —GW7647 dose response under optimized conditions



PPAR alpha UAS-*bla* HEK 293T cells were assayed in three separate experiments. Transduced cells were thawed and plated at 20,000 cells/well in a 384-well format the day of the assay. After a 3 hour pre-incubation, cells were stimulated with GW7647 (Calbiochem #370698) for 16-18 hours in the presence of 0.1% DMSO. 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 loading times were obtained using a standard fluorescence plate reader in bottom-read mode, and the background-corrected Response Ratios plotted against the indicated concentrations of GW7647 (n=16 for each data point).

Alternate Ligand Dose Response

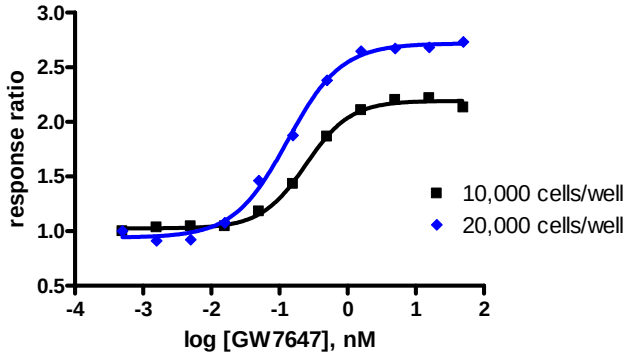
Figure 2 —Dose Response of other PPAR compounds



PPAR alpha UAS-*bla* HEK 293T cells (10,000 cells/well) were freshly trandsuced prior to assay and plated the day of the assay in a 384-well format. Cells were stimulated with either GW1929, rosiglitazone, L-165,041, GW9662, TO070907, GW6471, GW7647, GW501516, and GW0742 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 2 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 Ratios plotted against the indicated concentrations of the agonists (n= 4 for each data point).

Assay Performance with Variable Cell Number

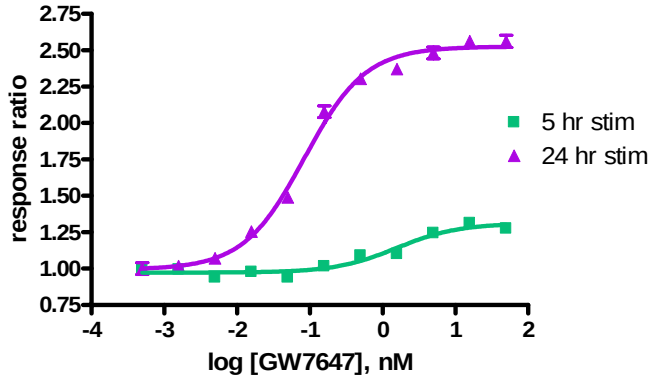
Figure 3— GW7647 dose response with 10k or 20K cells / well



PPAR alpha UAS-*bla* HEK 293T cells were thawed and plated at 10,000 or 20,000 cells/well in a 384-well format the day of the assay. After a 3 hour pre-incubation, cells were stimulated with GW7647 (Calbiochem #370698) for 16-18 hours in the presence of 0.1% DMSO. 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 in bottom-read mode, and the background-corrected Response Ratios plotted against the indicated concentrations of GW7647 (n=16 for each data point).

Assay performance with Variable Stimulation Time

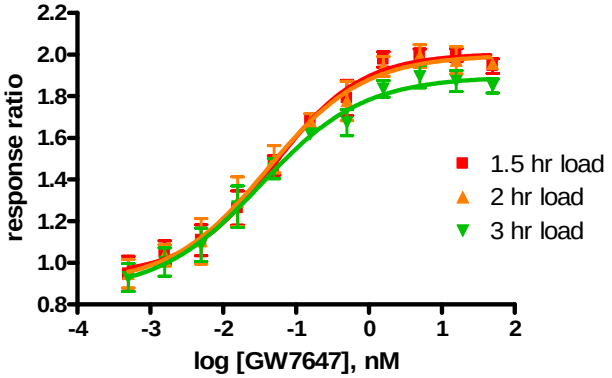
Figure 4 – GW7647 dose response with 5 and 24 hour stimulation times



PPAR alpha UAS-*bla* HEK 293T cells were thawed and plated at 10,000 cells/well in a 384-well format the day of the assay. Cells were immediately stimulated with GW7647 (Calbiochem #370698) for 5 or 24 hours in the presence of 0.1% DMSO. 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 in bottom-read mode, and the background-corrected Response Ratios plotted against the indicated concentrations of GW7647 (n=16 for each data point).

Assay performance with Variable Substrate Loading Time

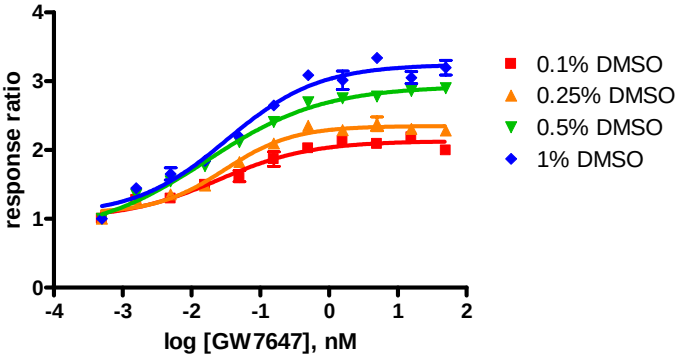
Figure 5 – GW7647 dose response with 1.5, 2, and 3 hour loading times



PPAR alpha UAS-*bla* HEK 293T cells were thawed and plated at 10,000 cells/well in a 384-well format the day of the assay. Cells were immediately stimulated with GW7647 (Calbiochem #370698) for 16-18 hours in the presence of 0.1% DMSO. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for either 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 plotted against the indicated concentrations of GW7647 (n=4 for each data point).

Assay performance with Variable DMSO concentration

Figure 6 – GW7647 dose response with 0.1-1% DMSO



PPAR alpha UAS-*bla* HEK 293T cells were thawed and plated at 10,000 cells/well in a 384-well format the day of the assay. After a 1.5 hour pre-incubation, cells were stimulated with GW7647 (Calbiochem #370698) for 16-18 hours in the presence of 0.1%, 0.25%, 0.5%, or 1.0% DMSO. 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 in bottom-read mode, and the background-corrected Response Ratios plotted against the indicated concentrations of GW7647 (n=4 for each data point).

References

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