GeneBLAzer® HCRTR2-Gα15 CHO-K1 DA Assay Kit

GeneBLAzer® HCRTR2-Gα15-NFAT-*bla* CHO-K1 Cells

Catalog Numbers – K1369 and K1737

Cell Line Descriptions

GeneBLAzer® HCRTR2-Gα15 CHO-K1 DA (Division Arrested) cells and GeneBLAzer® HCRTR2-Gα15-NFAT-*bla* CHO-K1 cells contain the human Hypocretin 2 receptor (HCRTR2), (Accession # NM_001526) stably integrated into the GeneBLAzer® Ga15-NFAT-*bla* CHO-K1 cell line. Gα15-NFAT-*bla* CHO-K1 cells (Cat. No. K1537) contain a beta-lactamase (*bla*) reporter gene under control of the nuclear factor of activated T-cells Response Element (NFAT), as well as the promiscuous G-protein, Ga15. Division Arrested (DA) cells are available as 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 GeneBLAzer® HCRTR2-Gα15 CHO-K1 DA cells and GeneBLAzer® HCRTR2-Gα15-NFAT-*bla* CHO-K1 cells are functionally validated for Z'-factor and EC_{50} concentrations of Orexin B (Figure 1). In addition, GeneBLAzer® HCRTR2-Gα15-NFAT-*bla* CHO-K1 cells have been tested for assay performance under variable conditions, including DMSO concentration, cell number, stimulation time, and substrate loading time. Additional testing data using alternate stimuli are also included.

Target Description

Orexin peptides (also referred to as hypocretins) were cloned in 1998 in two separate studies (1, 2). Orexin A and B are 46% homologous, derived from a precursor protein called preproorexin, and contain 33 and 28 amino acids, respectively (1, 2). Orexins are produced mainly in the hypothalamus, but other expressing cells have been found in other areas of the brain, spinal cord (3-5), neurons in the gut and stomach, and adrenal glands (reviewed in 6).

Two receptors responsive to orexin peptides have been identified as OX1 and OX2 (also known as HCRTR1 and HCRTR2). These receptors were found to be 64% identical and have different affinities for the orexin peptides. HCRTR2 has high affinity for both orexin peptides while HCRTR1 binds Orexin A more specifically. Expression of these receptors has been detected in the brain (1) as well as adrenal gland, gastrointestinal tract, and pancreas (reviewed in 6). The signaling cascades used by orexin receptors appear to be varied and cell-type dependant. In CHO cells, calcium release is seen after agonist treatment (1). In hypothalamic neurons, the frequency of action potentials is increased (2). Studies in human fetal adrenal glands report HCRTR2 coupling to Gi and Gs proteins (7) while an additional study in adult adrenal cells reports Gi, Gs, or Gq coupling (8).

Administering either orexin peptide into the bloodstream of rats has been shown to cause an increase in feeding behavior (1). The orexin system is also thought to regulate neuroendocrine function (9, 10) and the sleep wake cycle (10). HCRTR2 has been directly implicated in narcolepsy in animal studies (11-13).

Validation Results

Performance of this assay was evaluated under various conditions in 384-well format using LiveBLAzer™-FRET B/G Substrate.

1. **Orexin B agonist dose response under optimized conditions**

	DA cells	Dividing Cells
EC_{50}	1.4 nM	5.3 nM
Z'-factor	0.86	0.89

Optimum cell no.	= 20K cells/well
Optimum [DMSO]	= up to 1%
Optimum Stim. Time	= 5 hours
Max. [Stimulation]	= 200nM

2. **Alternate agonist dose response**

Orexin A	EC_{50}	= 3.1 nM

3. **Antagonist dose response**
No commercial antagonists were available at the time of publication of this document

4. **2nd messenger dose response**

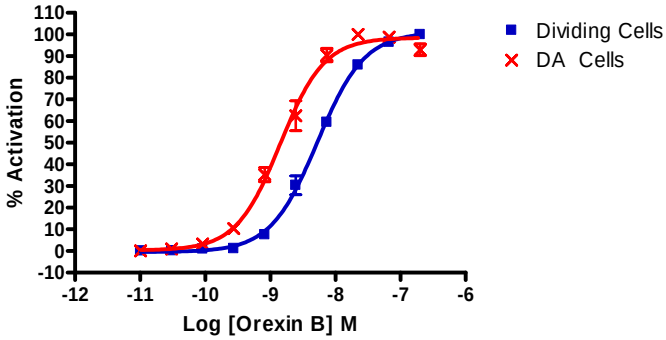
Orexin B	EC_{50}	= 907 pM

Assay Performance with Variable Conditions

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

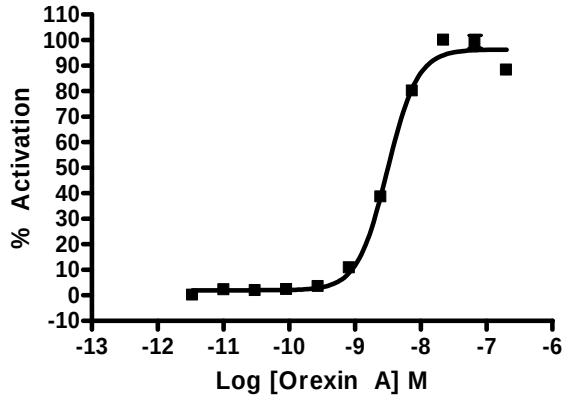
Figure 1 — GeneBLAzer® HCRTR2-Gα15 CHO-K1 DA and GeneBLAzer® HCRTR2-Gα15-NFAT-*bla* CHO-K1 dose response to Orexin B under optimized conditions



GeneBLAzer® HCRTR2-Gα15 CHO-K1 DA cells and GeneBLAzer® HCRTR2-Gα15-NFAT-*bla* CHO-K1 cells (10,000 cells/well) were plated in a 384-well format and incubated for 16-20 hours. Cells were stimulated with a dilution series of Orexin B in the presence of 0.5% DMSO for 5 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 2 hours. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and % Activation plotted for each replicate against the concentrations of Orexin B (n=6 for each data point).

Alternate Agonist Dose Response

Figure 2 — GeneBLAzer® HCRTR2-Gα15-NFAT-*bla* CHO-K1 dose response to Orexin A



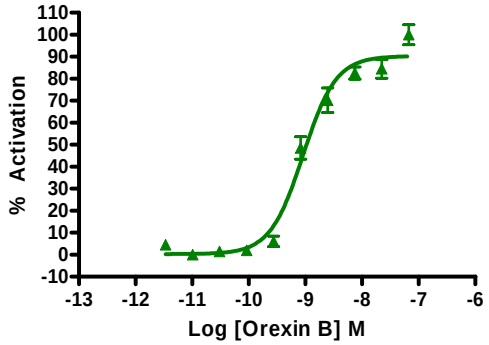
GeneBLAzer® HCRTR2-Gα15-NFAT-*bla* CHO-K1 cells (10,000 cells/well) were plated the day before the assay in a 384-well format. Cells were stimulated with Orexin A (Phoenix Pharmaceuticals #003-30) over the indicated concentration range in the presence of 0.5% DMSO for 5 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 2 hours. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the % Activation plotted against the indicated concentrations of agonist (n=16 for each data point).

Antagonist Dose Response

There were no commercial sources of antagonist available for testing at the time of publication of this document.

Agonist 2nd Messenger Dose Response

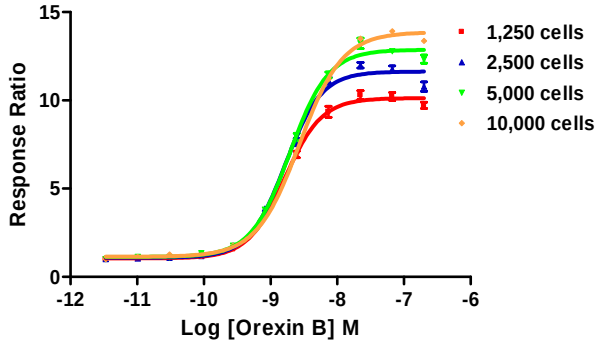
Figure 3— GeneBLAzer® HCRTR2-Gα15-NFAT-*bla* CHO-K1 2nd messenger dose response to Orexin B under optimized conditions



GeneBLAzer® HCRTR2-Gα15-NFAT-*bla* CHO-K1 cells were loaded with Fluo4-AM and tested for a response to Orexin B.

Assay Performance with Variable Cell Number

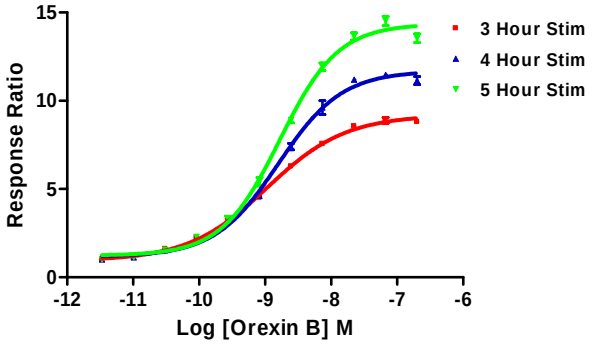
Figure 4— GeneBLAzer® HCRTR2-Gα15-NFAT-*bla* CHO-K1 dose response to Orexin B using 1.25, 2.5, 5, and 10K cells/well



GeneBLAzer® HCRTR2-Gα15-NFAT-*bla* CHO-K1 cells were plated the day before the assay at 1,250 2,500 or 5,000 and 10,000 cells/well in a 384-well format. On the day of the assay, cells were stimulated with Orexin B (Sigma #O6137) in the presence of 0.5% DMSO for 5 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate 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 for each cell number plotted against the indicated concentrations of Orexin B (n=8 for each data point).

Assay Performance with Variable Stimulation Time

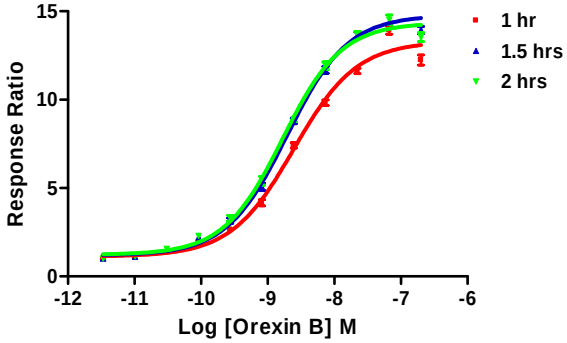
Figure 5 – GeneBLAzer® HCRTR2-Gα15-NFAT-*bla* CHO-K1 dose response to Orexin B with 3, 4 and 5 hr stimulation times



GeneBLAzer® HCRTR2-Gα15-NFAT-*bla* CHO-K1 cells (10,000 cells/well) were plated the day before the assay in a 384-well assay plate. Orexin B (Sigma #O6137) was then added to the plate over the indicated concentration range for 3, 4, or 5 hrs in 0.5% DMSO and 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 Response Ratios for each substrate loading time plotted against the indicated concentrations of Orexin B (n=8 for each data point).

Assay Performance with Variable Substrate Loading Times

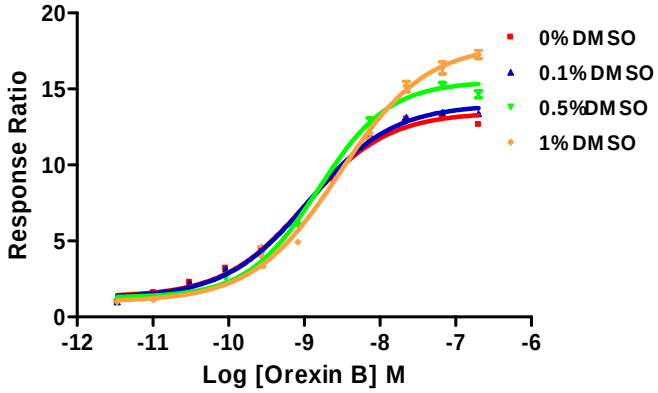
Figure 6— GeneBLAzer® HCRTR2-Gα15-NFAT-*bla* CHO-K1 dose response to Orexin B with 1, 1.5, and 2 hour substrate loading times.



GeneBLAzer® HCRTR2-Gα15-NFAT-*bla* CHO-K1 cells (10,000 cells/well) were plated the day before the assay in a 384-well black-walled tissue culture assay plate. Orexin B (Sigma #O6137) was then added to the plate over the indicated concentration range in 0.5% DMSO for 5 hours and then loaded for 1, 1.5 or 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 Response Ratios for each substrate loading time plotted against the indicated concentrations of Orexin B (n=8 for each data point).

Assay Performance with Variable DMSO Concentration

Figure 7 – GeneBLAzer® HCRTR2-Gα15-NFAT-*bla* CHO-K1 dose response to Orexin B with 0, 0.1, 0.5 and 1% DMSO



GeneBLAzer® HCRTR2-Gα15-NFAT-*bla* CHO-K1 cells (10,000 cells/well) were plated the day before the assay in a 384-well assay plate. Orexin B (Sigma #O6137) was then added to the plate over the indicated concentration range. DMSO was added to separate wells at concentrations from 0% to 1%. Cells were stimulated for 5 hrs with agonist and 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 Response Ratios for each DMSO concentration plotted against the indicated concentrations of Orexin B (n=8 for each data point).

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