GeneBLAzer® EDG3-Gα15 HEK 293T DA Assay Kit

GeneBLAzer® EDG3 -Gα15-NFAT-*bla* HEK 293T Cells

Catalog Number – K1319 and K1713

Cell Line Descriptions

GeneBLAzer® EDG3-Gα15 HEK 293T DA (Division Arrested) cells and GeneBLAzer® EDG3-Gα15-NFAT-*bla* HEK 293T cells contain the human EDG3 receptor (Accession # NM_005226) stably integrated in the GeneBLAzer® Gα15-NFAT-*bla* HEK 293T cell line. GeneBLAzer® Gα15-NFAT-bla HEK 293T cells (#K1539) contain a beta-lactamase (*bla*) reporter gene under control of the Nuclear Factor of Activated T-cells (NFAT) response element, and stably express the promiscuous G-protein, Gα15. 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® EDG3-Gα15 HEK 293T DA cells and GeneBLAzer® EDG3-Gα15-NFAT-*bla* HEK 293T cells are functionally validated for Z'-factor and EC_{50} concentrations of Sphingosine-1-phosphate (S1P), (Figure 1). In addition, GeneBLAzer® EDG3-Gα15-NFAT-*bla* HEK 293T 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

S1P is a bioactive lysophospholipid with diverse biological functions. It is a key cell signaling molecule that has been shown to act as both an intracellular second messenger and an extracellular ligand for a related group of five GPCRs in the endothelial differentiation gene (EDG) family of receptors (1,2). S1P is a polar sphingolipid metabolite that is derived through the multi-step enzymatic metabolism of the abundant membrane phospholipid, sphingomyelin (3). S1P is found at high nM levels in human serum and plasma where it is bound extensively by albumin and other plasma proteins (3, 4). Most of the S1P in the blood is released from activated platelets (5), while a small percentage of circulating S1P is released by other blood cells (6).

EDG-3(Endothelial-differentiation-gene-3)/S1P-3(Sphingosine-1-Phosphate-3) is a Gq/Gi/Go/Gα13/ Gα12 coupled GPCR. EDG-3 has been shown to be responsible for bradycardia that has been induced in clinical trials as a side effect of the immunosuppressive drug FTY720 that targets EDG-1 (7). EDG-3 has also been shown to induce vasodilation in response to sphingosylphosphorylcholine (SPC), sphingosine-1-phosphate (S1P), and lysosulfatide (LSF) (8). The EDG-3 Receptor has also been shown to play a cooperative or redundant role in angiogenesis with EDG-1(9).

Validation Summary

Development and Assessment of this assay was evaluated in a 384-well format using LiveBLAzer™-FRET B/G Substrate.

1. **S1P agonist dose response under optimized conditions**

	DA cells	Dividing Cells
EC_{50}	3.9 nM	3.8 nM
Z'-Factor	0.82	0.65

Optimum cell no.	= 5K cells/well
Optimum [DMSO]	= up to 0.5%
Optimum Stim. Time	= 5 hours
Max. [Stimulation]	= 10µM

2. **Alternate agonist dose response**

dh-S1 EC_{50}	= 5.6 nM

3. **Antagonist dose response**

CAY10444 IC_{50}	= 4.6 µM

4. **Agonist 2nd messenger response**

S1P EC_{50}	= 31 nM

Assay Development Summary

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

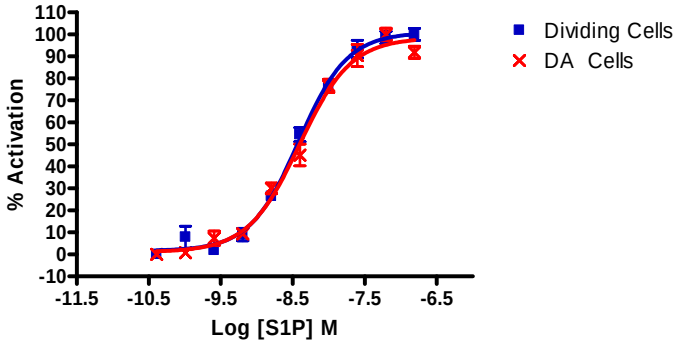
6. **Assay performance with variable stimulation time**

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

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

Primary Agonist Dose Response

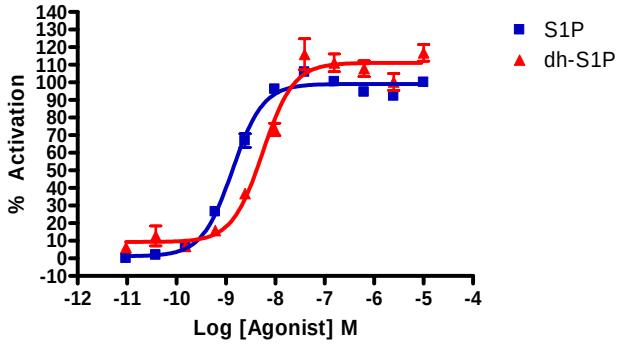
Figure 1 — GeneBLAzer® EDG3-Gα15 HEK 293T DA and EDG3-Gα15-NFAT-*bla* HEK 293T dose response to S1P under optimized conditions



GeneBLAzer® EDG3 HEK 293T DA cells and GeneBLAzer® EDG3-Gα15-NFAT-*bla* HEK 293T 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 S1P 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 S1P (n=6 for each data point).

Alternate Agonist Dose Response

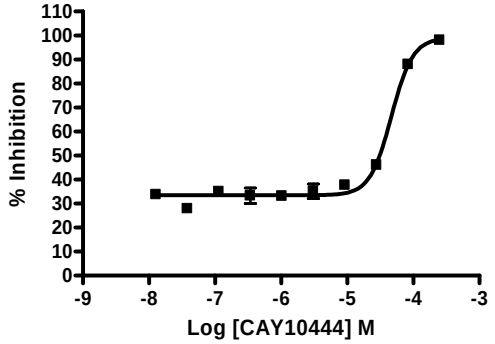
Figure 2 — GeneBLAzer® EDG3-Gα15-NFAT-*bla* HEK 293T dose response to S1P and dh-S1P under optimized conditions



GeneBLAzer® EDG3-Gα15-NFAT-*bla* HEK 293T cells (5,000 cells/well) were plated the day before the assay in a 384-well format. On the day of the assay, cells were stimulated with S1P (Avanti Polar Lipids #860492P) and dh-S1P (Avanti Polar Lipids #860536P) as a 4-fold dilution series from a maximum stimulation concentration of 10µM to a minimum concentration of 38pM 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 S1P and dh-S1P.

Antagonist Dose Response

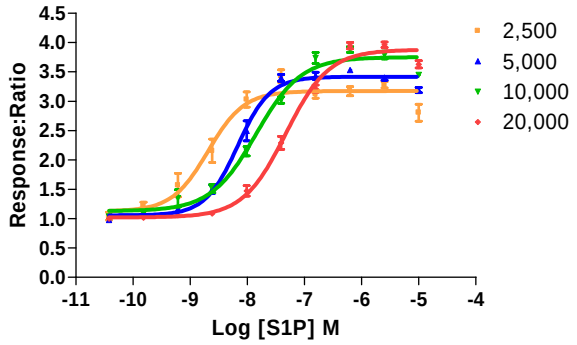
Figure 3 — GeneBLAzer® EDG3-Gα15-NFAT-*bla* HEK 293T dose response to CAY10444



GeneBLAzer® EDG3-Gα15-NFAT-*bla* HEK 293T cells were plated the day before the assay at 5,000 cells per well in 384-well format. Cells were treated with CAY10444 (Cayman Chemical #10005033) as a 3-fold dilution series from a maximum concentration of 250µM to a minimum concentration of 12.7nM and incubated at 37ºC & 5% CO_2 for 45 min. S1P was added to the plate at the EC_{80} concentration of 15.7nM and cells were incubated at 37ºC & 5% CO_2 for 5 hours 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 % Inhibition shown plotted against the indicated concentrations of the antagonist. CAY10444 is an EDG-3 selective antagonist.

Assay Performance with Variable Cell Number

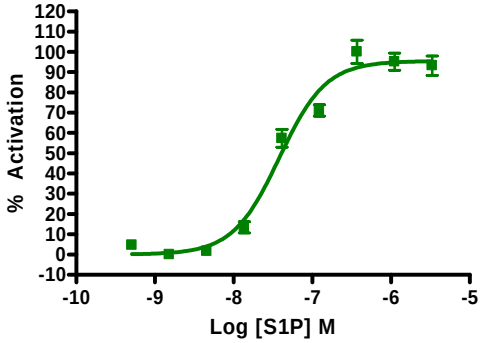
Figure 5 — GeneBLAzer® EDG3-Gα15-NFAT-*bla* HEK 293T dose response to S1P with 2.5, 5, 10 and 20K cells/well



GeneBLAzer® EDG3-Gα15-NFAT-*bla* HEK 293T cells were plated the day before the assay at 2,500 5,000 10,000 or 20,000 cells/well in a 384-well format. On the day of the assay, cells were stimulated with S1P (Avanti Polar Lipids #860492P) 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 plotted against the indicated concentrations of S1P (n=8 for each data point).

Agonist 2nd Messenger Response

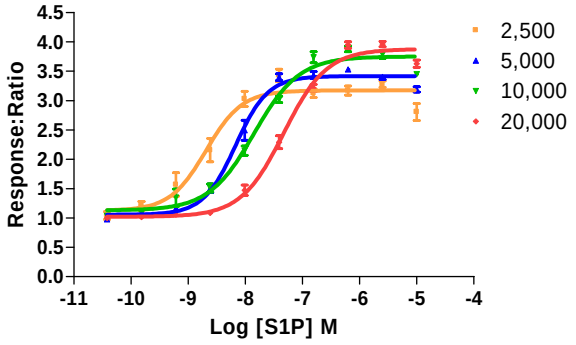
Figure 4 — GeneBLAzer® EDG3-Gα15-NFAT-*bla* HEK 293T dose response to S1P using Fluo-4NW



GeneBLAzer® EDG3-Gα15-NFAT-*bla* HEK 293T cells (5,000 cells/well) were plated the day before the assay in a 384-well format. Cells were then incubated with Fluo-4NW for 30 min at 37°C., followed by 30 min. at room temperature. Cells were then stimulated with a dilution series of S1P (Avanti Polar Lipids #860492P) in the presence of 0.5% DMSO. Fluorescence emission values at 516 nm were obtained and plotted against the indicated concentrations of S1P (n=16 for each data point).

Assay Performance with Variable Cell Number

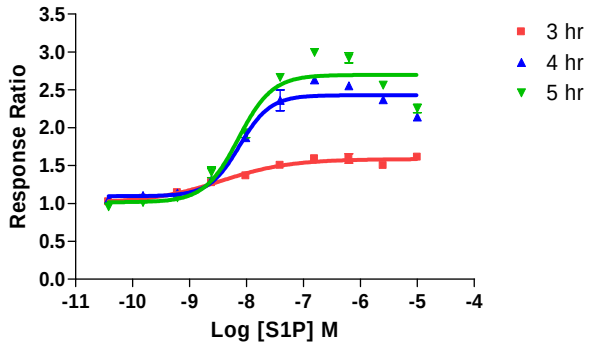
Figure 5 — GeneBLAzer® EDG3-Gα15-NFAT-*bla* HEK 293T dose response to S1P with 2.5, 5, 10 and 20K cells/well



GeneBLAzer® EDG3-Gα15-NFAT-*bla* HEK 293T cells were plated the day before the assay at 2,500 5,000 10,000 or 20,000 cells/well in a 384-well format. On the day of the assay, cells were stimulated with S1P (Avanti Polar Lipids #860492P) 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 plotted against the indicated concentrations of S1P (n=8 for each data point).

Assay Performance with Variable Stimulation Time

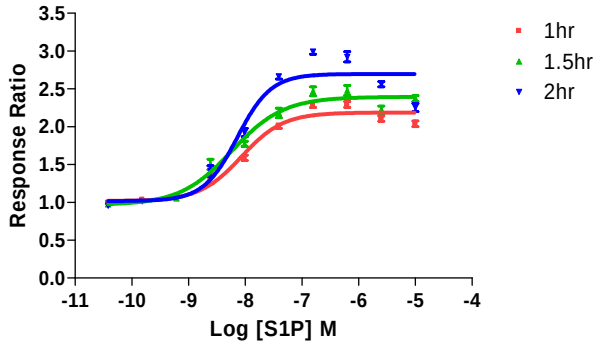
Figure 6 – GeneBLAzer® EDG3-Gα15-NFAT-*bla* HEK 293T dose response to S1P with 3, 4 and 5 hour stimulation times



GeneBLAzer® EDG3-Gα15-NFAT-*bla* HEK 293T cells (5,000 cells/well) were plated the day before the assay in a 384-well format. S1P (Avanti Polar Lipids #860492P) was then added to the plate as a 4-fold dilution series from a maximum stimulation concentration of 10µM to a minimum concentration of 38pM. Plates were stimulated for 3, 4, or 5 hrs with S1P 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 for each stimulation time using a standard fluorescence plate reader and the Response Ratios plotted against the indicated concentrations of S1P (n=16 for each data point).

Assay Performance with Variable Substrate Loading Time

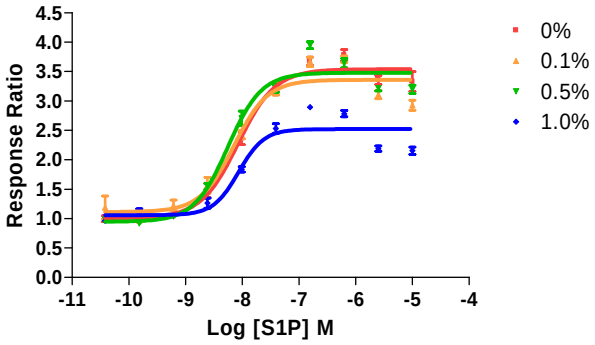
Figure 7 – GeneBLAzer® EDG3-Gα15-NFAT-*bla* HEK293T dose response to S1P with 1, 1.5 and 2 hr substrate loading times



GeneBLAzer® EDG3-Gα15-NFAT-*bla* HEK 293T cells (5,000 cells/well) were plated the day before the assay in a 384-well format. S1P (Avanti Polar Lipids #860492P) was then added to the plate as a 4-fold dilution series from a maximum stimulation concentration of 10µM to a minimum concentration of 38pM. Plates were stimulated for 5 hrs with S1P in 0.5% DMSO 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 for each substrate loading time using a standard fluorescence plate reader and the Response Ratios plotted against the indicated concentrations of S1P (n=16 for each data point).

Assay Performance with Variable DMSO Concentration

Figure 8 – GeneBLAzer® S1P dose response with 0, 0.1, 0.5 and 1% final DMSO concentrations.



GeneBLAzer® EDG3-Gα15-NFAT-*bla* HEK 293T cells (5,000 cells/well) were plated the day before the assay in a 384-well format. S1P (Avanti Polar Lipids #860492P) was then added to the plate as a 4-fold dilution series from a maximum stimulation concentration of 10µM to a minimum concentration of 38pM. DMSO was added to the assay at concentrations from 0% to 1%. Plates were stimulated for 5 hrs 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 plotted for each DMSO concentration against the indicated concentrations of S1P (n=8 for each data point).

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