GeneBLAzer® M5 CHO-K1 DA Assay Kit

GeneBLAzer® M5 NFAT-*bla* CHO-K1 Cells

Catalog Numbers – K1351 and K1728

Cell Line Descriptions

GeneBLAzer® M5 CHO-K1 DA (Division Arrested) cells and GeneBLAzer® M5-NFAT-*bla* CHO-K1 cells contain the human Acetylcholine (muscarinic) subtype 5 (M5) receptor (Accession #BC041805.1) stably integrated into the CellSensor® NFAT-*bla* CHO-K1 cell line. CellSensor® NFAT-*bla* CHO-K1 cells (Cat. no. K1534) contain a beta-lactamase (*bla*) reporter gene under control of the Nuclear Factor of Activated T-cells (NFAT) response element. 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® M5 CHO-K1 DA cells and GeneBLAzer® M5-NFAT-*bla* CHO-K1 cells are functionally validated for Z'-factor and EC_{50} concentrations of carbachol (Figure 1). In addition, M5-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

Muscarinic acetylcholine receptors are members of the G protein-coupled receptor (GPCR) superfamily. Muscarinic receptors are widely distributed and mediate the actions of acetylcholine in both the CNS and peripheral tissues. Five muscarinic receptor subtypes have been identified and are referred to as M_1-M_5 (1-5).

The M_1, M_3, and M_5 receptor subtypes couple through the $G_{q/11}$ class of G-proteins and activate the phopholipase C pathway. Activation of this pathway in turn leads to increases in free intracellular calcium levels as inositol triphosphate mediates release of calcium from the endoplasmic reticulum. In addition, protein kinase C is activated via diacylglycerol. The M_2 and M_4 receptor subtypes couple through the $G_{i/o}$ class of G proteins and inhibit adenylyl cyclase activity.

The M_5 subtype is expressed in brain at very low levels with a limited distribution. The physiological relevance of this receptor subtype remains unknown, primarily because of its low expression levels and the lack of M_5 receptor-selective ligands. However, knockout studies have suggested that the role of M_5 involves modulation of central dopamine function and the tone of cerebral blood vessels (6-8). In addition, M_5 may be important in the treatment of addiction (8). Additional information on the muscarinic receptors can be found in reviews (9-12).

Validation Summary

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

1. Carbachol agonist dose response under optimized conditions

	DA cells	Dividing Cells
EC_{50}	0.5 µM	1.0 µM
Z'-factor	0.94	0.94

Optimum cell no.	= 10K cells/well
Optimum [DMSO]	= up to 1%
Optimum Stim. Time	= 3 hours
Max. [Stimulation]	= 30 µM

2. Alternate agonist dose response

Oxotrmorine M EC_{50}	= 193 nM
MCN–A-343 EC_{50}	= 9.4 µM
Pilocarpine EC_{50}	= 17 µM
Bethanecol EC_{50}	= 25 µM

3. Antagonist dose response

Scopolamine IC_{50}	= 2 nM
DAMP IC_{50}	= 6 nM
Telenzipine IC_{50}	= 23 nM
Methoctramine IC_{50}	= 18 µM

4. Agonist Dose Response Using Fluo-4NW

Carbachol EC_{50}	= 508 nM

Assay Testing Summary

5. Assay performance with variable cell number

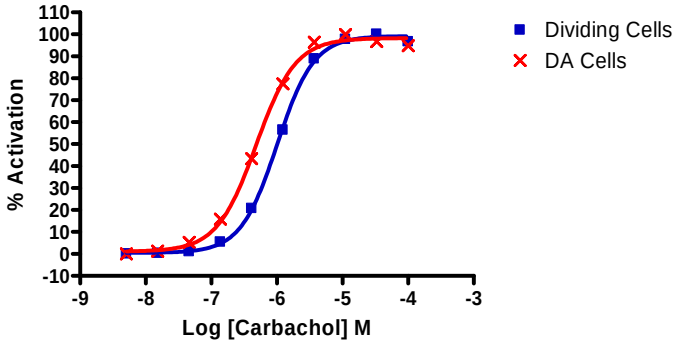
6. Assay performance with variable stimulation time

7. Assay performance with variable substrate loading time

8. Assay performance with variable DMSO concentration

Primary Agonist Dose Response

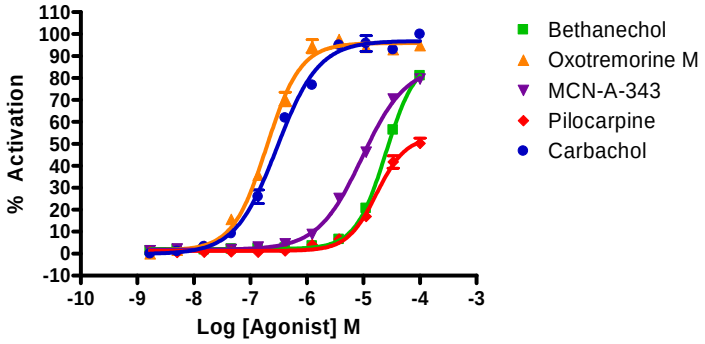
Figure 1 — GeneBLAzer® M5 CHO-K1 DA and M5-NFAT-*bla* CHO-K1 dose response to carbachol under optimized conditions



GeneBLAzer® M5 CHO-K1 DA cells and GeneBLAzer® M5-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 carbachol 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 carbachol (n=6 for each data point).

Alternate Agonist Dose Response

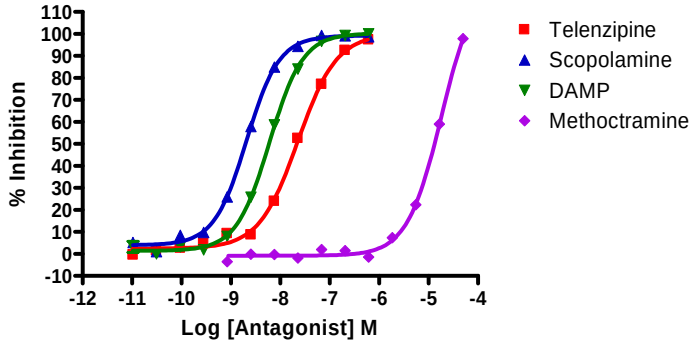
Figure 2 — GeneBLAzer® M5-NFAT-*bla* CHO-K1 dose response to Bethanecol, Oxotremorine M, MCN-A-343, Pilocarpine and Carbachol



GeneBLAzer® M5-NFAT-*bla* CHO-K1 cells (20,000 cells/well) were plated the day of the assay in a 384-well format. Cells were stimulated with Carbachol (Sigma #21760), Bethanechol (Sigma #C5259), Oxotremorine (Sigma #O-100), MCN-A-343 (Sigma #C7041), or Pilocarpine (Sigma #P6503) 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 shown plotted against the indicated concentrations of the agonists.

Antagonist Dose Response

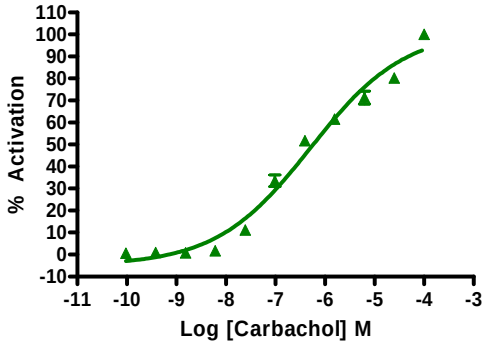
Figure 3 — GeneBLAzer® M5-NFAT-*bla* CHO-K1 dose response to Telenzipine, Scopolamine, DAMP and Methoctramine



GeneBLAzer® M5-NFAT-*bla* CHO-K1 cells (20,000 cells/well) were plated the day of the assay in a 384-well black-walled tissue culture assay plate. Cells were treated with the antagonists Telenzipine (T-122), Scolpolamine (S1875), DAMP (D104) and Methoctramine (M-105) and incubated at 37 degrees C for 20 min., followed by 1.5 µM Carbachol agonist stimulation for 5 hours in 0.5% DMSO. 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 % Inhibition was plotted against the indicated concentrations of the antagonist. The data shows the correct rank order potency.

Agonist Dose Response Using Fluo-4NW

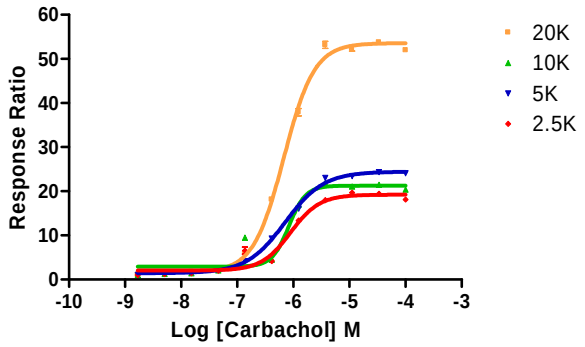
Figure 4 — GeneBLAzer® M5-NFAT-*bla* CHO-K1 dose response to Carbachol using Fluo-4NW



GeneBLAzer® M5-NFAT-*bla* CHO-K1 cells (10,000 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 Carbachol (Sigma #21760) in the presence of 0.5% DMSO. Fluorescence emission values at 516 nm were obtained and the % Activation plotted against the indicated concentrations of carbachol (n=16 for each data point).

Assay Performance with Variable Cell Number

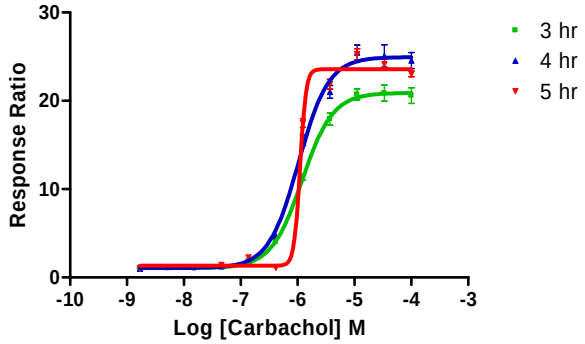
Figure 5— GeneBLAzer® M5-NFAT-*bla* CHO-K1 dose response using 2.5, 5, 10, and 20K cells/well



GeneBLAzer® M5-NFAT-*bla* CHO-K1 cells were plated at 2,500, 5000, 10,000 or 20,000 cells/well in a 384-well format. On the day of the assay, cells were stimulated with Carbachol (Sigma #21760) 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 Carbachol (n=8 for each data point).

Assay performance with Variable Stimulation Time

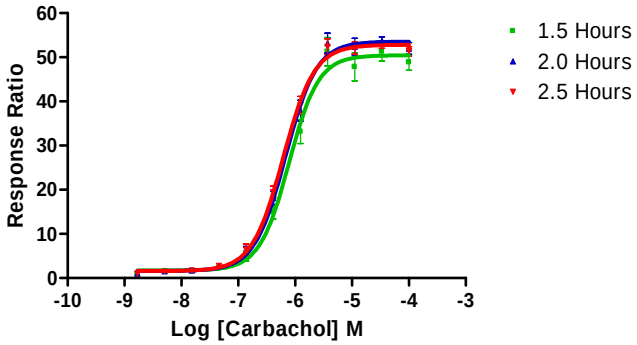
Figure 6 – GeneBLAzer® M5-NFAT-*bla* CHO-K1 dose response using 3, 4, and 5 hour stimulation times



GeneBLAzer® M5-NFAT-*bla* CHO-K1 cells (20,000 cells/well) were plated the day of the assay in a 384-well black-walled tissue culture assay plate. Carbachol (Sigma #21760) 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 were plotted against the indicated concentrations of carbachol (n=8 for each data point)

Assay performance with Variable Substrate Loading Time

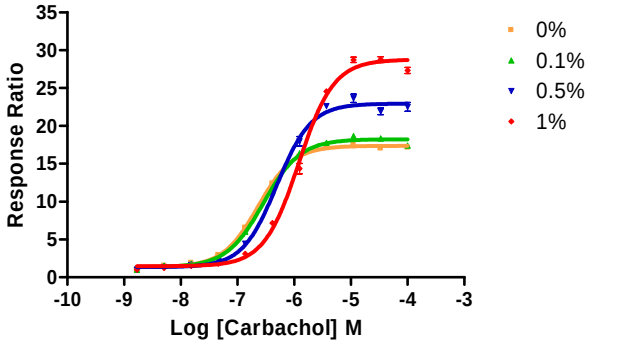
Figure 7 – GeneBLAzer® M5-NFAT-*bla* CHO-K1 dose response using 1.5, 2, and 2.5 hr substrate loading times



GeneBLAzer® M5-NFAT-*bla* CHO-K1 cells were plated at 20,000 cells/well in a 384-well format. On the day of the assay, cells were stimulated with Carbachol (Sigma #21760) in the presence of 0.5% DMSO for 5 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for either 1.5, 2 or 2.5 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 Carbachol (n=8 for each data point).

Assay Performance with variable DMSO concentration

Figure 8 – GeneBLAzer® M5-NFAT-*bla* CHO-K1 dose response using 0, 0.1, 0.5 and 1% DMSO.



GeneBLAzer® M5-NFAT-*bla* CHO-K1 cells (10,000 cells/well) were plated the day of the assay in a 384-well black-walled tissue culture assay plate. Carbachol (Sigma #21760) 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 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 are for each DMSO concentration were plotted against the indicated concentrations of carbachol (n=8 for each data point).

References

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