GeneBLAzer® M1 CHO-K1 DA Cells

GeneBLAzer® M1 NFAT-*bla* CHO-K1 Cells

Catalog Numbers – K1365 and K1735

Cell Line Descriptions

GeneBLAzer® M1 CHO-K1 DA (Division Arrested) cells and GeneBLAzer®M1-NFAT-*bla* CHO-K1 cells contain the human Acetylcholine (muscarinic) subtype 1 receptor (M1), (Accession #NM_000738) 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 in 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® M1 CHO-K1 DA cells and GeneBLAzer® M1-NFAT-*bla* CHO-K1 cells are functionally validated for Z'-factor and EC_{50} concentrations of carbachol (Figure 1). In addition, M1-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 five genes that encode the muscarinic receptors all belong to the rhodopsin-line family (Family A) and share strong sequence homology but have unique regions located at the amino terminus (extracellular) and in the third intracellular loop.

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.

In the brain, M_1 activation mediates "slow" neuronal excitability. Cortical and hippocampal muscarinic receptors are thought to be important in the attentional aspects of cognition. The predominant receptor subtypes in these brain areas are M_1, M_3, and M_4. Therefore, M_1 is a potential target for cognition, Alzheimer's, dementia, and schizophrenia (6). Studies on knock-out mouse models of M_1 are also beginning to reveal potential functions of the receptor (7-9). Additional information on the muscarinic receptors can be found in reviews (10-13).

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}	165 nM	105 nM
Z'-factor	0.92	0.87

Optimum cell no.	= 20K cells/well
Optimum [DMSO]	= up to 1%
Optimum Stim. Time	= 5 hours
Max. [Stimulation]	= 10 µM

2. Alternate agonist dose response

Bethanecol EC_{50}	= 3 µM
Oxotrmorine M EC_{50}	= 3.8 nM
MCN –A-343 EC_{50}	= 162 nM
Pilocarpine EC_{50}	= 366 nM

3. Antagonist dose response

Telenzipine IC_{50}	= 10 nM
Scopolamine IC_{50}	= 2.2 nM
DAMP IC_{50}	= 22 nM
Methoctramine IC_{50}	= 3.1 µM

4. Agonist Dose Response Using Fluo-4NW

Carbachol EC_{50}	= 55 nM

5. Agonist Dose Response with ERK2 Phoshporylation

Methacholine EC_{50}	= 74.3 nM
Oxotrmorine M EC_{50}	= 9.8 nM
MCN –A-343 EC_{50}	= 208 nM
Carbachol EC_{50}	= 20.9 nM

6. [^{3}H] NMS saturation binding analysis

K_D [^{3}H] NMS	= 0.1 nM
B_{max} (pmol/mg)	= 2.0

7. Allosteric Modulation

Assay Testing Summary

8. Assay performance with variable cell number

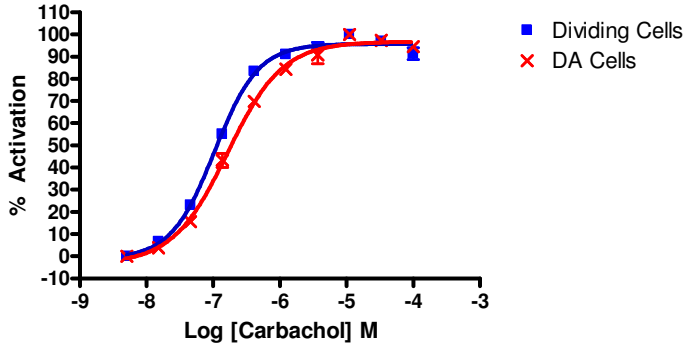
9. Assay performance with variable stimulation time

10. Assay performance with variable substrate loading time

11. Assay performance with variable DMSO concentration

Primary Agonist Dose Response

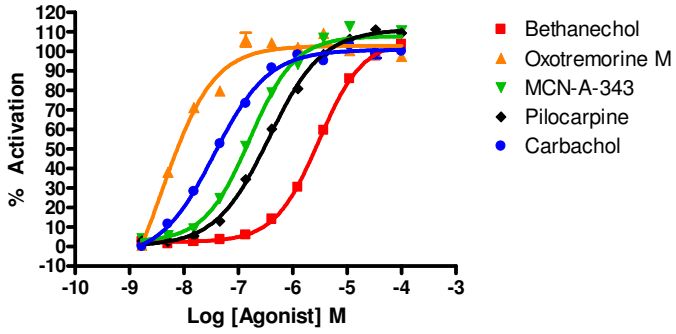
Figure 1 — GeneBLAzer® M1-NFAT-*bla* CHO-K1 DA and GeneBLAzer® M1-NFAT-*bla* CHO-K1 dose response to carbachol under optimized conditions



GeneBLAzer® M1-NFAT-*bla* CHO-K1 DA cells and GeneBLAzer® M1-NFAT-*bla* CHO-K1 cells (20,000 cells/well) were plated the day of the assay in a 384-well format and stimulated with Carbachol (Sigma #21760) 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 carbachol (n= 4 for each data point).

Alternate Agonist Dose Response

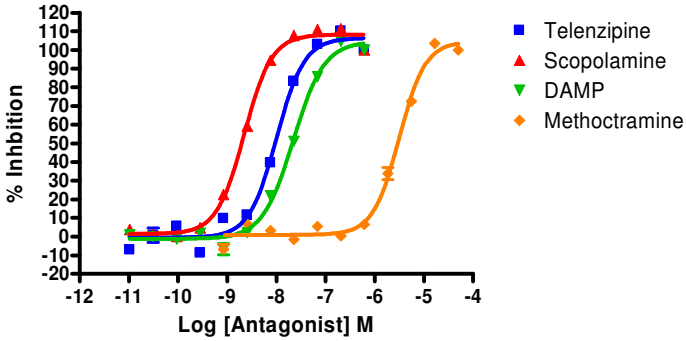
Figure 2 — GeneBLAzer® M1-NFAT-*bla* CHO-K1 dose response to Bethanecol, Oxotremorine M, MCN-A-343, Pilocarpine and Carbachol



GeneBLAzer® M1-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 either 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 plotted against the indicated concentrations of the agonists (n= 8 for each data point).

Antagonist Dose Response

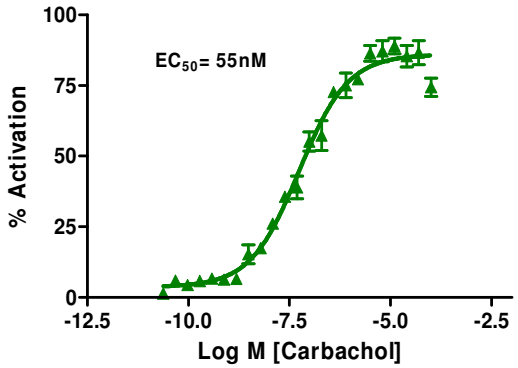
Figure 3 — GeneBLAzer® M1-NFAT-*bla* CHO-K1 dose response to Telenzipine, Scopolamine, DAMP and Methoctramine



GeneBLAzer® M1-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 telenzipine (Sigma #T-122), Scopolamine (Sigma #S1875), DAMP (Sigma #D104), or Methoctramine (Sigma #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 is shown plotted against the indicated concentrations of the antagonists. The data shows the correct rank order potency.

Agonist Dose Response Using Fluo-4NW

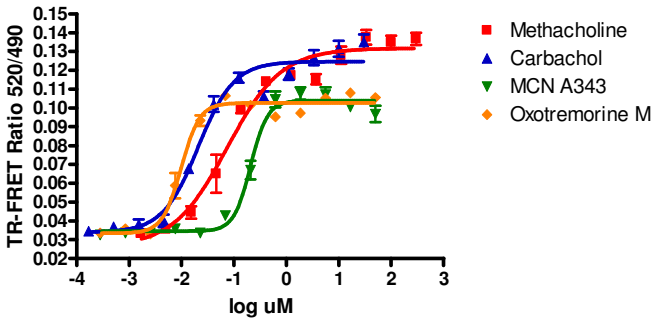
Figure 4 — GeneBLAzer® M1-NFAT-*bla* CHO-K1 dose response to Carbachol using Fluo-4NW



GeneBLAzer® M1-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 plotted as % Activation against the indicated concentrations of carbachol (n=16 for each data point).

ERK2 Phosphorylation

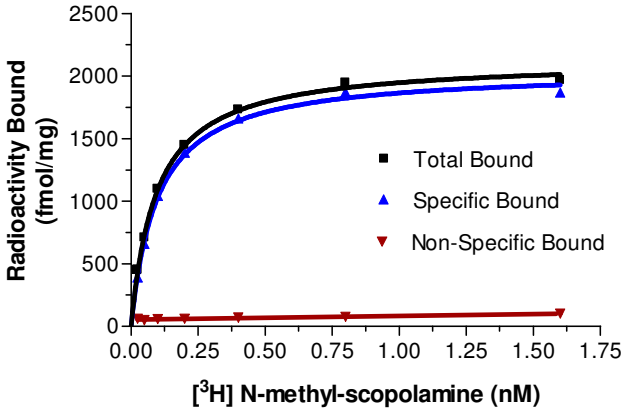
Figure 5 — GeneBLAzer® M1-NFAT-*bla* CHO-K1 agonist dose responses with the BacMam ERK2 [pThr185/pTyr187] Cellular Assay Kit



GeneBLAzer® M1-NFAT-*bla* CHO-K1 cells were assayed with the BacMam ERK2 [pThr185/pTyr187] Cellular Assay Kit. Transduced cells were stimulated for 6 minutes with a dilution series of either Methacholine, Carbachol, MCN-A-343, or Oxotremorine M prior to detection of ERK2 phosphorylation with the TR-FRET kit. Time resolved fluorescence emission values at 520 nm and 490 nm were obtained using a standard fluorescence plate reader and the TR-FRET ratio plotted.

Radioligand Binding

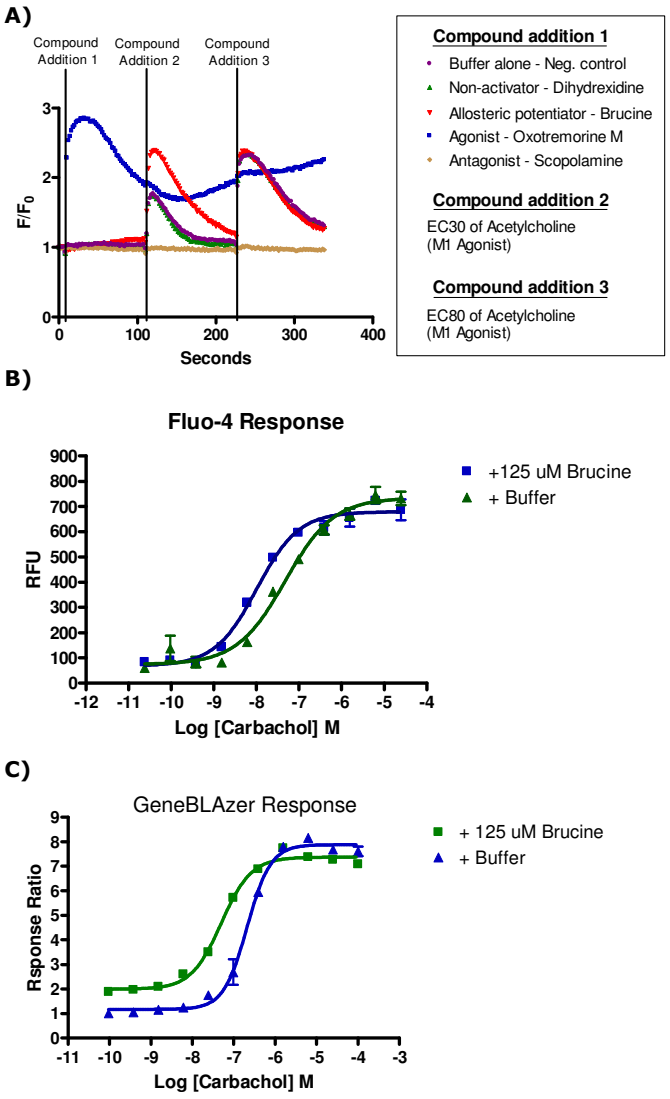
Figure 6 — [^{3}H] NMS saturation binding to GeneBLAzer® M1-NFAT-*bla* CHO-K1 Membranes



Saturation binding analysis was performed by incubating GeneBLAzer® M1 NFAT-*bla* CHO-K1 membranes with increasing concentrations of [^{3}H] N-methyl-scopolamine (NMS) in PBS, pH 7.4, with 100 µg/ml BSA. Non-specific binding was determined in the presence of 10 µM atropine. Samples were incubated for 2 hrs prior to filtration through a GF/B 96-well filter plate, which was pre-treated with 0.5% PEI. Filters were washed with cold 50 mM Tris-Cl, pH 7.4.

Allosteric Modulation

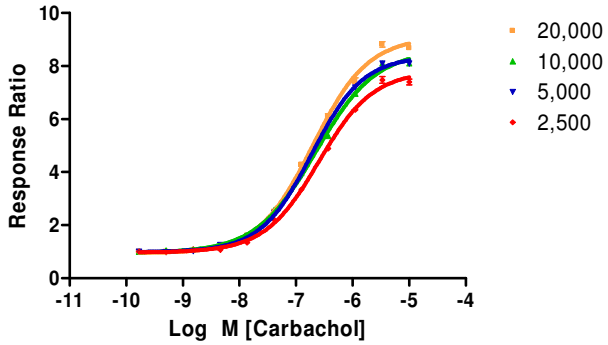
Figure 7 — Allosteric modulation of GeneBLAzer® M1-NFAT-*bla* CHO-K1 detected with Fluo-4 or the GeneBLAzer® Assay



GeneBLAzer® M1-NFAT-*bla* CHO-K1 were assayed with either Fluo-4 (Figures A and B) or beta-lactamase (Figure C). Brucine is an allosteric modulator of the M1 muscrinic receptor which when applied allows carbachol to have greater potency at lower doses.

Assay Performance with Variable Cell Number

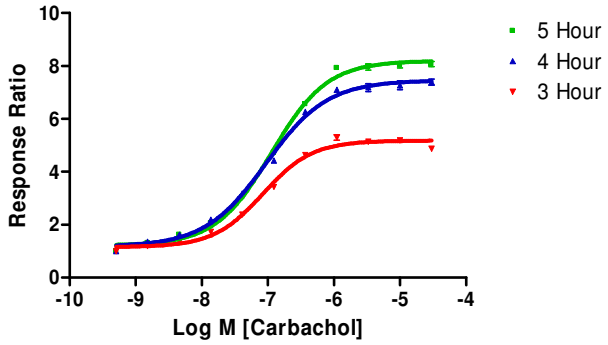
Figure 8— GeneBLAzer® M1-NFAT-*bla* CHO-K1 dose response to carbachol using 2.5, 5, 10, and 20K cells/well



GeneBLAzer® M1-NFAT-*bla* CHO-K1 cells were plated at 2500, 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 2.5 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 Stimulation Time

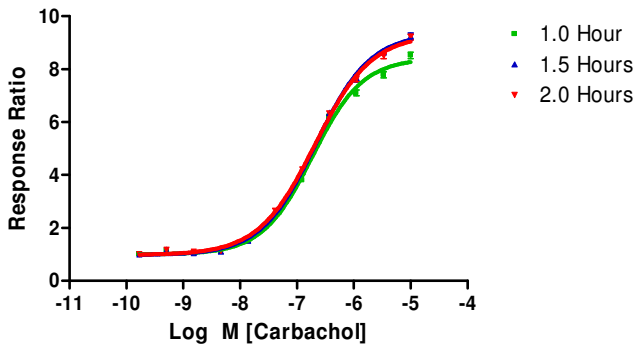
Figure 9 – GeneBLAzer® M1-NFAT-*bla* CHO-K1 dose response to carbachol using 3, 4, and 5 hour stimulation times.



GeneBLAzer® M1-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 plotted (n=8 for each data point)

Assay performance with Variable Substrate Loading Time

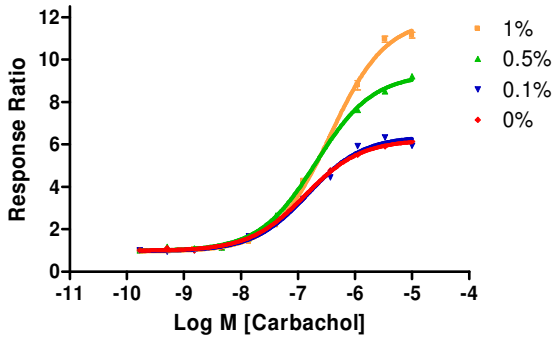
Figure 10 – GeneBLAzer® M1-NFAT-*bla* CHO-K1 dose response to carbachol using 1, 1.5, and 2 hour loading times



GeneBLAzer® M1-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, 1.5, and 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 DMSO concentration

Figure 11 – GeneBLAzer® M1-NFAT-*bla* CHO-K1 dose response to carbachol using 0, 0.1, 0.5 and 1% DMSO.



GeneBLAzer® M1-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 shown plotted for each DMSO concentration against the indicated concentrations of carbachol (n=8 for each data point).

References

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