**GeneBLAzer® M4-Gqo5 CHO-K1 DA Assay Kit**

**GeneBLAzer® M4-Gqo5 NFAT-*bla* CHO-K1 Cells**

Catalog Numbers – K1377 and K1741

## Cell Line Descriptions

GeneBLAzer® M4-Gqo5 CHO-K1 DA (Division Arrested) cells and GeneBLAzer®M4-Gqo5-NFAT-*bla* CHO-K1 cells contain the human Acetycholine (muscarinic) subtype 4 receptor (M4), (Accession #NM_000741) stably integrated into the Gqo5-NFAT-bla CHO-K1 cell line. Gqo5-NFAT-bla CHO-K1 cells (Cat. No. K1536) contain a beta-lactamase (*bla*) reporter gene under control of the Nuclear Factor of Activated T Cells (NFAT) response element and the chimeric G protein, Gqo5. 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®M4-Gqo5 CHO-K1 DA cells and GeneBLAzer®M4-Gqo5-NFAT-*bla* CHO-K1 cells are functionally validated for Z'-factor and $EC_{50}$ concentrations of carbachol, (Figure 1). In addition, GeneBLAzer®M4-GQO5-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. One downstream target activated by the phospholipase C pathway is the transcription factor, NFAT. The $M_2$ and $M_4$ receptor subtypes couple through the $G_{i/o}$ class of G proteins and inhibit adenylyl cyclase activity.

The clinical implications of the $M_4$ receptor are unknown; however, studies with knockout mice suggest that $M_4$ may have implications in dopaminergic function, karatinocyte migration and wound healing, anxiolysis, and analgesia (6-11). In addition, $M_4$ may be important in memory circuits and have implications in Alzheimer's Disease (12). Additional information on the muscarinic receptors can be found in reviews (13-16).

## 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 |
|---|---|---|
| $EC_{50}$ | 139 nM | 176 nM |
| Z'-factor | 0.64 | 0.60 |

Recommended cell no. = 10K cells/well
Recommended [DMSO] = up to 1%
Recommended Stim. Time = 5 hours
Max. [Stimulation] = 3 μM

### 2. Alternate agonist dose response

Oxotrmorine M $EC_{50}$ = 31 nM
MCN–A-343 $EC_{50}$ = 844 nM
Bethanecol $EC_{50}$ = 25 μM
Pilocarpine $EC_{50}$ = 66 μM

### 3. Antagonist dose response

Scopolamine $IC_{50}$ = 374 pM
Telenzipine $IC_{50}$ = 3.3 nM
DAMP $IC_{50}$ = 5.1 nM
Methoctramine $IC_{50}$ = 769 nM

### 4. Agonist Dose Response Using Fluo-4NW

Carbachol $EC_{50}$ = 406 nM

## Assay Testing Summary

### 5. Assay performance with variable cell number

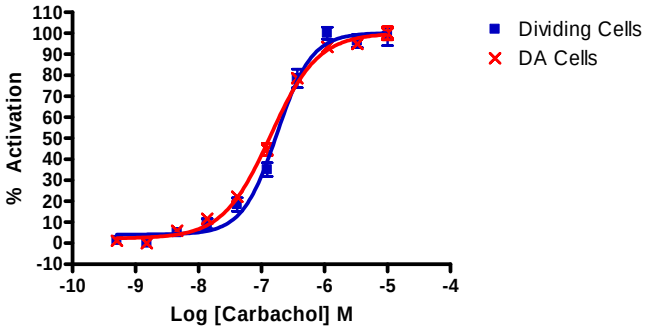
### 6. Assay performance with variable stimulation time

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

### 8. Assay performance with variable DMSO concentration

## Primary Agonist Dose Response

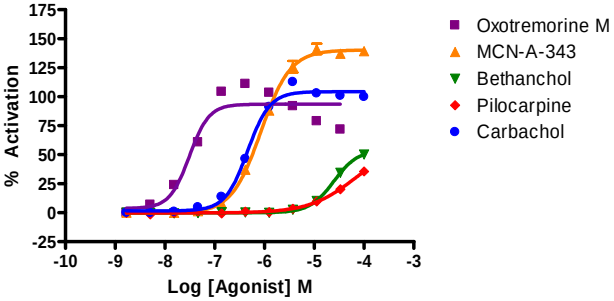
**Figure 1 — GeneBLAzer® M4-Gqo5 CHO-K1 DA and M4-Gqo5-NFAT-*bla* CHO-K1 dose response to carbachol under optimized conditions**




GeneBLAzer® M4-Gqo5 CHO-K1 DA cells and GeneBLAzer® M4-Gqo5-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 (2μM final concentration of CCF4-AM + 1mM Solution D) 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

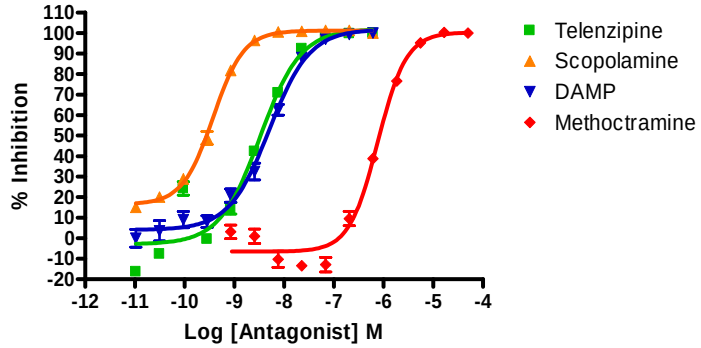
**Figure 2 — GeneBLAzer® M4-Gqo5-NFAT-*bla* CHO-K1 dose response to Bethanecol, Oxotremorine M, MCN-A-343, Pilocarpine and Carbachol**




GeneBLAzer® M4-Gqo5-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 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 is shown plotted against the indicated concentrations of the agonists.

## Antagonist Dose Response

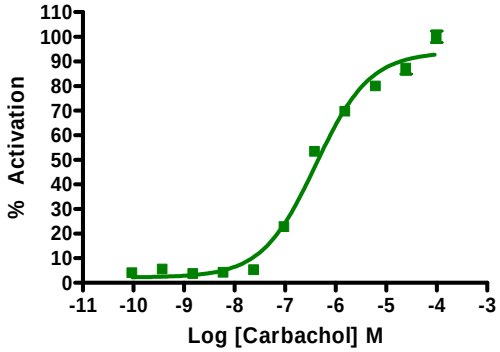
**Figure 3 — GeneBLAzer® M4-Gqo5-NFAT-*bla* CHO-K1 dose response to Telenzipine, Scopolamine, DAMP and Methoctramine**




GeneBLAzer® M5-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. 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 florescence 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

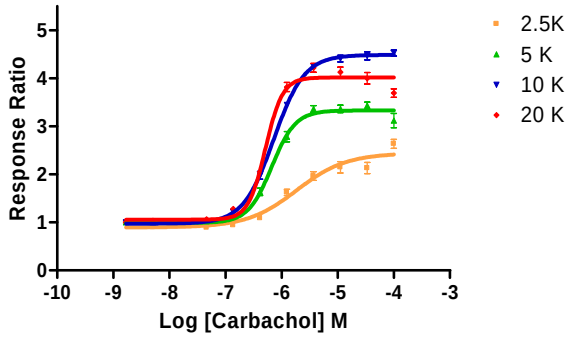
**Figure 4 — GeneBLAzer® M4-Gqo5-NFAT-*bla* CHO-K1 dose response to carbachol using Fluo-4NW**




GeneBLAzer® M4-Gqo5-NFAT-*bla* CHO-K1 cells (10,000 cells/well) were plated the day before the assay in a 384-well format. Cells were 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

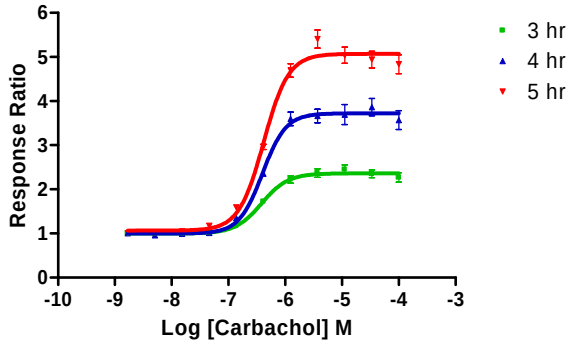
**Figure 5— GeneBLAzer® M4-Gqo5-NFAT-*bla* CHO-K1 dose response to Carbachol with 2.5, 5, 10, and 20K cells/ well**




GeneBLAzer® M4-Gqo5-NFAT-*bla* CHO-K1 cells were plated one day before the assay 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 (2µM final concentration of CCF4-AM + 1mM Solution D) 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

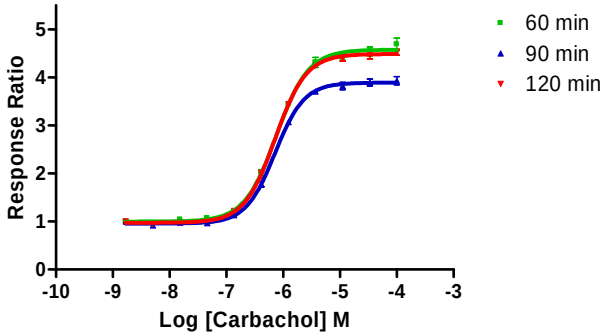
**Figure 6 – GeneBLAzer® M4-Gqo5-NFAT-*bla* CHO-K1 dose response to Carbachol with 3, 4, and 5 hour stimulation times**




GeneBLAzer® M4-Gqo5-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. 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(2µM final concentration of CCF4-AM + 1mM Solution D). Fluorescence emission values at 460 nm and 530 nm were obtained using a standard florescence 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

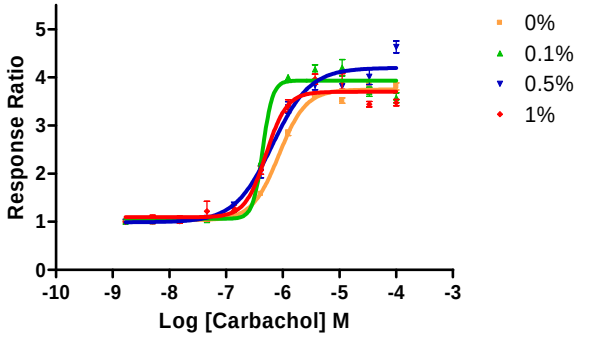
**Figure 7 – GeneBLAzer® M4-Gqo5-NFAT-*bla* CHO-K1 dose response to Carbachol with 1, 1.5, and 2 hr substrate loading times**




GeneBLAzer® M4-Gqo5-NFAT-*bla* CHO-K1 cells were plated at 10,000 cells/well in a 384-well format one day before the assay. 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 (2µM final concentration of CCF4-AM + 1mM Solution D) for either 1, 1.5, or 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 8 – GeneBLAzer® M4-Gqo5-NFAT-*bla* CHO-K1 dose response to Carbachol with 0, 0.1, 0.5 and 1% DMSO.**




GeneBLAzer® M4-Gqo5-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. 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 (2µM final concentration of CCF4-AM + 1mM Solution D). Fluorescence emission values at 460 nm and 530 nm were obtained using a standard florescence 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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