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ChemiScreen™ GLOW AEQUORIN CALCIUM-OPTIMIZED STABLE CELL LINE HUMAN RECOMBINANT M_1 MUSCARINIC ACETYLCHOLINE RECEPTOR

CATALOG NUMBER:	HTS044AG	**QUANTITY:**	2 vials, 1 mL per vial
LOT NUMBER:		**CONCENTRATION:**	2 x 10^6 cells/mL

BACKGROUND: The muscarinic acetylcholine receptor family consists of five GPCRs that mediate some of the neurotransmission functions of acetylcholine in the CNS and the periphery. The M_1 receptor, along with the M_3 and M_5 receptors, signal through $G_{q/11}$ and subsequent release of Ca^{++} from the ER. The M_1 receptor is expressed in ganglia and mediates depolarization of ganglia by inhibition of voltage-gated M-type K^+ channels. In addition, the M1 receptor mediates venous contraction (Caulfield and Birdsall, 1998). M_1-null mutant mice display increased locomotor activity accompanied by selected cognitive deficits, and are resistant to pilocarpine-induced epileptic seizures (Wess, 2004). Millipore's cloned human M_1-expressing cell line is made in the CHO-K1 host which stably expresses a mitochondrially targeted glow mutant form of aequorin. This glow variant of aequorin has shown a higher luminescent signal intensity than the original aequorin in vitro. Thus, the cell line is an ideal tool for screening for agonists and antagonists for M_1.

APPLICATIONS: Luminescent and fluorescent calcium flux assays

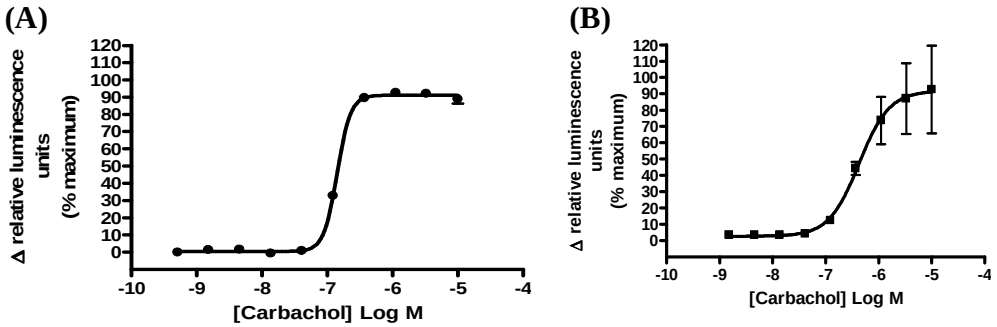

Figure 1. Ligand-induced calcium flux in Glow Aequorin CHO cell line stably transfected with M_1. Glow Aequorin CHO-K1 cell line stably co-expressing M_1 were loaded with 5 µM coelenterazine for 3 h at room temperature. Luminescence in response to carbamoylcholine was determined (A) in quadruplicate in a 384 well plate with a FLIPR^{TETRA} with aequorin option from (Molecular Devices, now part of MDS Analytical Technologies). Data were collected for area under curve for 240 sec. In this experiment, average maximum signal was 18,700 RLU (area under curve, AUC) and minimum was less than 250 RLU (AUC). Z' was 0.78 with 48 wells each buffer or carbamoylcholine at EC80. (B) Luminescence in response to carbamoylcholine was determined in duplicate in a 96 well plate with a Perkin Elmer Wallac Victor2. In this experiment, average maximum signal was 72,700 RLU (area under curve, AUC) and minimum was less than 2500 RLU (AUC) Data were collected for area under curve for 20 sec.

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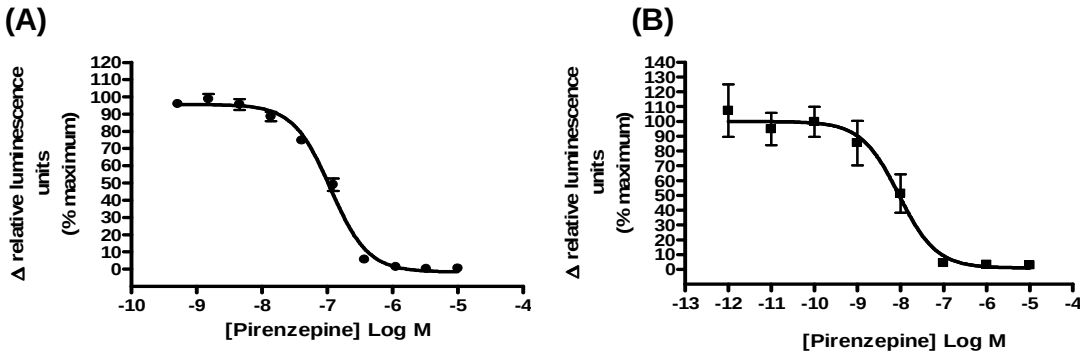

Figure 2. Assay for antagonist activity on ligand-induced calcium flux in Glow Aequorin CHO-K1 cell line stably transfected with M_1. Glow Aequorin CHO-K1 stably co-expressing M_1 were loaded with 5 μM coelenterazine for 3 h at room temperature. Pirenzapine was added to the cells at the final concentration indicated, and incubated for 10min at room temperature. (A) Luminescence in response to carbachol (2x EC50 concentration) was determined in quadruplicate in a 384 well plate with a FLIPR^{TETRA} system with aequorin option (Molecular Devices, now part of MDS Analytical Technologies). Data were collected for area under curve for 240 sec. (B) Luminescence in response to carbachol (2x EC50 concentration) was determined in duplicate in a 96 well plate with a Perkin Elmer Wallac Victor2. Data were collected for area under curve for 20 sec.

Table I. Comparison of EC50 values of M_1- and glow aequorin-coexpressing CHO cells with values described in the literature.

ligand	assay	Mean potency (nM)	Reference
Carbamoylcholine	Luminescent calcium	EC50 = 157	Figure 1a (FLIPR^{TETRA})
Carbamoylcholine	Luminescent calcium	EC50 = 500	Figure 1b (Wallac Victor2)
Carbamoylcholine	Fluorescent calcium	EC50 = 7	Millipore HTS044C datasheet
Carbamoylcholine	Calcium	EC50 = 1700	Connors and Ruzicka, 1999
Carbamoylcholine	Calcium	EC50 = 4.2	Langmead *et al*., 2006
Pirenzepine	Calcium	IC50 = 10.8	Figure 2a (FLIPR^{TETRA})
Pirenzepine	Calcium	IC50 = 9.1	Figure 2b (Wallac Victor2)
Pirenzepine	Calcium	K_B = 7.4	Langmead *et al*., 2006
Pirenzepine	[^{35}S]-GTPγS binding	K_B = 22	Lazareno and Birdsall, 1993

HOST CELLS: CHO-K1

TRANSFECTION: Plasmids containing full-length human CHRM1 cDNA endocing M1

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(Accession Number: NM_000738) and enhanced glow aequorin with mitochondrial targeting sequence. The stable clonal cell line was selected by resistance to geneticin and hygromycin, followed by limited dilution cloning. The cell line was tested and found to have equivalent EC50 and signal:background >20 at 1, 3 and 6 weeks of continuous culture.

PRESENTATION:

Cells are frozen at 2 x 10^6 cells/mL in 90% heat inactivated fetal bovine serum /10% DMSO. Cell line tests negative for mycoplasma.

STORAGE/HANDLING:

1. Immediately upon receipt, thaw cells or place cells in liquid nitrogen. Maintain frozen in liquid nitrogen for up to 5 years.
2. Thaw cells rapidly by removing from liquid nitrogen and immediately immersing in a 37ºC water bath. Immediately after ice has thawed, sterilize the exterior of the vial with 70% ethanol. Transfer contents of the vial to a T75 flask containing growth media. Place the flask in a humidified incubator at 37ºC with 5% CO_2.
3. After 8-24 h, all live cells will be attached. Viability of the cells is expected to be 50-80%. At this time, replace media to remove residual DMSO, and return to incubator.
4. When cells are approximately 80% confluent, passage the cells as follows: Remove media and wash once with HBSS without Ca^{++} and Mg^{++} (10 mL/T75). Add 0.05% trypsin/0.2 g/L EDTA (1 mL/T75) and place in humidified incubator at 37ºC with 5% CO_2 until cells begin to round up and detach (2-4 minutes). Gently rap the side of the flask to dislodge the cells. Neutralize trypsin by addition of 4 mL CHO Aequorin Growth Media per 1 mL trypsin.
5. Cells are typically passaged 1:10 every 3-4 days. Passaging ratio may be varied according to requirements of the investigator.
6. Frozen stocks of cells should be prepared at the earliest passage possible after thawing, as follows: Count detached cells (prepared as in Step 4). Centrifuge cells at 200 x g for 5 min. Resuspend cells at 5 x 10^6 cells/mL in Freezing Media (cell densities of 2-10 x 10^6 are also acceptable if necessary). Dispense 1 mL aliquots into cryopreservation vials. Freeze the cells by a controlled rate process, such as in an isopropanol-jacketed container placed at –70ºC overnight. Store the via ls in liquid nitrogen.
7. Use of cells immediately after thawing is feasible for some cell lines and is being further validated. Some cell lines may need to be passaged at least once after thawing prior to use in calcium flux assays.

MEDIA:

CHO Aequorin Growth Media:
F-12K Nutrient Mixture, Kaighn's Modification with 2 mM L-glutamine
10% heat inactivated fetal bovine serum
0.25 mg/ml Geneticin (G418)
0.2 mg/ml Zeocin
100 U/ml each penicillin and streptomycin (from 100x stock, Millipore TMS-AB2-C)

CHO Freezing Media
90% heat inactivated fetal bovine serum
10% DMSO

CHO Aequorin Plating Media:
F-12K Nutrient Mixture, Kaighn's Modification with 2 mM L-glutamine
10% heat inactivated fetal bovine serum
100 U/ml each penicillin and streptomycin

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RECOMMENDED ASSAY CONDITIONS:

1. Seed cells in 384-well opaque-walled, clear bottom plate overnight at 10,000 cells/well in CHO Aequorin Plating Media.

   Note: *Cells may also be loaded and assayed in suspension on the same day.*

2. Remove media and add 25 µL per well 5µM of coelenterazine (Millipore ES016) in Wash Buffer (HBSS with Ca^{++} and Mg^{++} containing 10 mM HEPES). Incubate at room temperature for 3 hours in the dark.

   Note: *Luminescence activity has been determined to be optimal at room temperature. Incubation at 37°C will result in substantially red uced signals.*

3. Analyze in luminescence mode in a $FLIPR^{TETRA}$ with Aequorin Option with the suggested gain setting of 140K.
4. If antagonists are to be analyzed, add 12.5 µL/well antagonist solution to the cells in the coelenterazine solution. Incubate for 10 min at room temperature in the dark.
5. Begin the luminescence reading on the FLIPR. The program is set to read for 240 sec, with 12.5 µL/well agonist solution added at 20 sec. Typically the glow luminescence peaks within 30 sec after ligand addition and has returned to baseline by 200 sec post addition.
6. Data may be analyzed as peak height or area under curve.

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