APPLICATION NOTE

AEQUOSCREEN PLASMIDS

AequoScreen aequorin-encoding Plasmids for performing aequorin luminescent assay starting from non-aequorin cell lines

Introduction

Calcium signaling and Aequorin

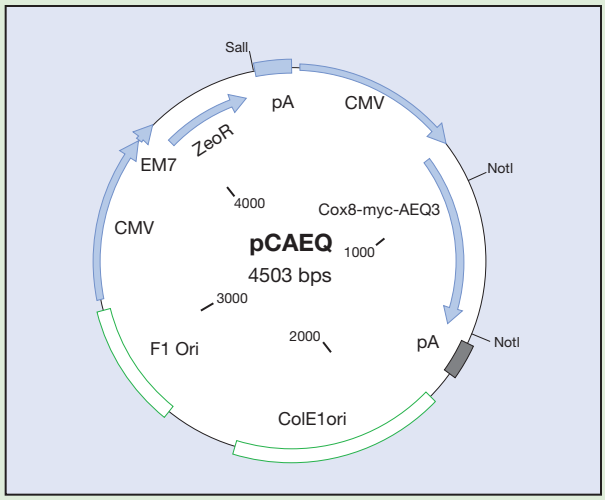
Aequorin is a photoprotein originating from the jellyfish *Aequorea Victoria* (Inouye *et al.*, Proc. Nat. Acad. Sci. USA 82, 3154-3158, 1985; Prasher *et al.*, Biochem. Biophys. Res. Commun. 126 :1259-1268, 1985). The apo-enzyme (apoaequorin) is a 21 kD protein, which requires a hydrophobic prosthetic group, coelenterazine, to be converted to aequorin, the active form of the enzyme. This enzyme possesses 3 calcium binding sites which control its activity. Upon calcium binding, aequorin oxidizes coelenterazine into coelenteramide with the production of CO_2 and emission of light. The consumption of aequorin is proportional to the calcium concentration within a physiological range (50 nM to 50 µM) (Brini *et al.*, J. Biol. Chem. 270: 9896-9903, 1995; Rizzuto *et al.*, Biochem. Biophys. Res. Commun. 126: 1259-1268, 1995). Therefore, measurement of the light emitted upon oxidation of coelenterazine is a reliable tool for measurement of intracellular calcium flux and furthermore generates results comparable to those obtained with traditional fluorescent dyes (Brini *et al.*, J. Biol. Chem. 270: 9896-9903, 1995).

Aequorin stable cell lines have traditionally been generated by transfection and co-expression of aequorin with a specific target of interest. The aequorin plasmids now offer the possibility to convert an existing stable recombinant cell line into a luminescence cell line or the possibility of performing dual transient transfections (i.e. aequorin plasmid and target receptor plasmid) into different host cell lines.

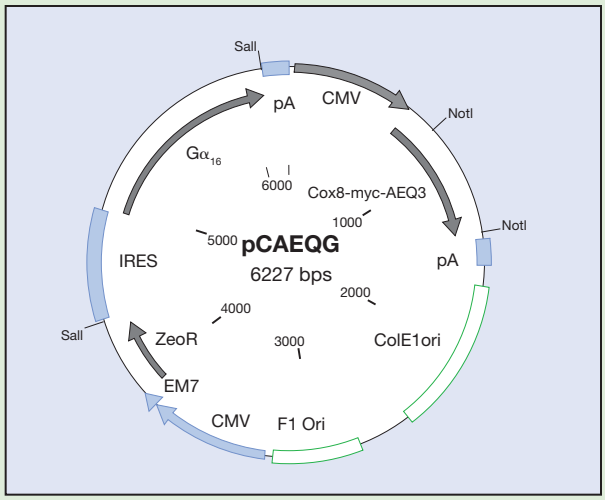
This application note demonstrates the possibility of transiently transfecting the aequorin AequoScreen® plasmid into GPCR recombinant stable cell lines. Luminescent properties obtained via this method were equivalent to results obtained by generating stable aequorin cell lines. Two aequorin AequoScreen plasmids, pCAeq and pCAeqG, encoding aequorin and aequorin with $G\alpha_{16}$, respectively, enable luminescent assays for GPCRs from the three main coupling families, i.e. G_s-, G_q- and G_i-coupled receptors.

Plasmid structure

Map of the pCAeq AequoScreen plasmid



Map of the pCAeqG AequoScreen plasmid



Structure of the pCAeq AequoScreen Plasmid

This plasmid is intended for expression of mitochondrially-targeted aequorin. Codons of the aequorin cDNA were optimized for expression in cells of human origin. The cDNA coding for mitochondrially expressed aequorin is under the control of a human cytomegalovirus immediate-early (CMV) promoter for high-level expression in a wide range of mammalian cells (Boshart *et al.*, 1985), while a second promoter is responsible for the expression of the Zeocin-resistance gene.

The apoaequorin encoded by this plasmid is a fusion protein between the mitochondrial-targeting sequence of cytochrome C oxidase subunit VIII and apoaequorin, as described by Rizzuto *et al.* (1992). The fusion protein also includes a myc epitope for detection of the fusion protein.

Structure of the pCAeqG AequoScreen Plasmid

This plasmid is intended for expression of both mitochondrially-targeted aequorin and the $G\alpha_{16}$ GTP-binding protein. Features of the pCAeqG plasmid were designed as described for pCAeq. The second promoter however is responsible for both the expression of the Zeocin-resistance gene and expression of $G\alpha_{16}$ via the use of an IRES sequence.

The $G\alpha_{16}$ protein, whose expression is naturally restricted to a subset of haematopoietic cells, has the remarkable property to be able to contact almost any GPCR, with very few exceptions, and to stimulate phospholipase C in response to the binding of an agonist of these receptors. This leads to the coupling of a lot of GPCRs to the calcium pathway in cell lines co-expressing the $G\alpha_{16}$ protein (Offermanns & Simon, 1995; Milligan *et al*, 1996).

Methods

Transient transfection

- The day before the experiment, detach the cells by gentle flushing in a trypsin solution.
- Centrifuge at 1500 g for 2 min, count cells and resuspend cells in culture medium (see table below) at a concentration of 4 x 10^5 cells/mL. Seed a Petri dish (10 cm diameter) with 10 mL of cell suspension.
- The day of the experiment, perform a visual inspection of the dishes, cells should be 90-95% confluent.
- Remove the medium and add 10 mL of Ham's F12 or DMEM or EMEM with FBS but without antibiotics.
- Prepare a 15 mL Falcon tube with the plasmid DNA in Opti-MEM medium, without serum or antibiotics. 12 µg DNA for 1.5 mL Opti-MEM. In a second tube, prepare 20 µL of Lipofectamine™ 2,000 in 1.5 mL of Opti-MEM.
- Incubate the two tubes 5 min at room temperature.
- Pool contents of tubes and mix (no vortex).

Cell Line	Recommended culture medium
CHO	Ham's F12 medium + 10% Fetal Bovine Serum + 100 IU/mL penicillin, 100 µg/mL streptomycin and G418 (400 µg/mL)
HEK293	EMEM medium with 2mM L-glutamine + 10% Fetal Bovine Serum + 100 IU/mL penicillin, 100 µg/mL streptomycin and G418 (100 µg/mL)
1321N1	DMEM medium + 10% Fetal Bovine Serum + 1mM sodium pyruvate + 100 IU/mL penicillin, 100 µg/mL streptomycin and G418 (500 µg/mL)

- Incubate for another 20 min at room temperature.
- After the incubation, add 3 mL of the mix to the Petri dish containing the cells.
- Incubate the cells during 30-48 hours at 37 °C, with 5% CO_2 before running the Aequorin assay.

Aequorin Assay

- The day before the assay, after visual inspection of the dishes/flasks, cells should be 70-80% confluent.
- Cells are detached by gentle flushing with PBS/5 mM EDTA.
- Cells are centrifuged, counted and resuspended at 1 x 10^6 cells/mL in BSA medium [DMEM/Ham's F12 (with 15 mM HEPES, L-glutamine, without phenol red) culture medium + 10% protease-free BSA in H_2O (final BSA concentration is 0.1%)] in a Falcon tube.
- Add Coelenterazine h at a final concentration of 5 µM in assay medium. As coelenterazine stock solution is in methanol, mix well while adding the coelenterazine solution to the cell suspension to avoid damaging the cells.
- The 10 mL Falcon tube is wrapped in aluminum foil and placed on a rotating wheel (about 45° angle and 7 rpm). Alternatively, cells can be gently agitated using a magnetic stirrer and a magnetic rod equipped with a ring to avoid crushing the cells.
- Cells are incubated 18 h at 20 °C (temperature should remain below 25 °C).
- On the day of the assay, dilute cells in BSA medium to a final concentration of 2.0 x 10^5 cells/mL and transfer to a beaker wrapped in aluminum foil on a magnetic stirrer. Use a stirring bar with a ring to avoid crushing the cells (low speed). For the MicroBeta® JET and the MicroLumat, the minimal volume needed is 50 mL (1 x 10^7 cells).
- Incubate the cells for at least 1 h at room temperature.
- Prepare the ligand plate. Ligands are diluted in BSA medium, and 50 µl are dispensed per well. In case DMSO is used for the stock of ligand, ensure that the final DMSO concentration in the assay plate does not exceed 10% in the case of an agonist assay, and does not exceed 1% in the case of an antagonist assay.

The value to take into consideration for data analysis is the integration of emitted light during the 20 seconds of measurement (i.e. area under the curve). The EC_{50} is determined as the concentration of a ligand which induces a response halfway between the baseline and maximum. The dose response curve is performed using 10 concentrations of ligand, each concentration being assayed in duplicate. The "Top/Digit" response (expressed in percentage) is the ratio between the response from the max ligand concentration (EC_{100}) of the receptor (Top) and the digitonin response (Digit), which is indicative of the aequorin content of the cells. When transiently transfecting the aequorin gene in cells already expressing a GPCR, this value is indicative of the quality of the coupling of the GPCR in the cells (depends on expression level and intrinsic coupling capacities), and not of the success of transfection by the aequorin gene.

The signal to background ("S/B"), is the ratio between the buffer response and the maximum of the ligand response. The Z′ and the CV values in agonist mode are calculated using the EC_{100} concentration of the reference agonist and the buffer response. The Z′ and CV values in antagonist mode are calculated using the EC_{80} concentration of the reference agonist and the IC_{100} concentration of the reference antagonist.

ASSAY	96-well format
Agonist assay	Dispense 50 µL, i.e.10,000 cells/well of cell suspension into 50 µL of agonist solution (ligand plate) which has been pre-dispensed into a white OptiPlate 96. Measure the light emitted for 20 s.
Antagonist assay	Dispense 50 µL, i.e.10,000 cells/well of cell suspension into 50 µL of antagonist solution (ligand plate) which has been pre-dispensed into a white OptiPlate 96. Incubate cells with antagonist for 15 min at room temperature. Dispense 50 µL of agonist (3 x EC_{80} concentration to get 1 x EC_{80} final concentration) into the mix of cells and antagonist and record the light emitted for 20 s.

See materials list on page 7.

Schedule of the experiment

Day 1	Day 2	Day 3	Day 4
Detach the cells and culture at 4 x 10^5 cell/mL	In medium without antibiotics, transiently transfect cells with Lipofectamine (20 µL) and 12 µg DNA	Cell recovery and ON coelenterazine loading at 1.0 x 10^6 cell/mL	Dilution at 2.0 x 10^5cell/mL and Aequorin assay

Results

The transient transfection performances of the two AequoScreen plasmids were observed with GPCRs from different G-protein coupling families (G_s, G_q and G_i). A stable GPCR aequorin cell line was included in each experiment to allow comparison of the transient and stable transfection data.

G_q-coupled receptors

The two model $G_{q/11}$ coupled receptors chosen were the CHO Neurotensin NTS_1 cell lines (ES-690-C and ES-690-A) and the 1321N1 Cholecystokinin CCK_2 cell lines (ES-531-C and ES-531-A). The summary of these experiments is presented in graphical data in Figures 1, 2 and in Tables A and B.

Agonists	**Neurotensin**				**[Lys^{8}-Lys^{9}]NT**		
	pEC_{50}± SD	S/B	Top/Digit%	Z'/CV	pEC_{50}± SD	S/B	Top/Digit%
Neurotensin NTS_1 AequoScreen cell line	11.65± 0.19	23.30	86.55	0.86/ 3.24	11.58± 0.09	23.65	87.06
CHO Neurotensin NTS_1 cell line transiently transfected with pCAeq	10.91± 0.24	30.72	52.79	0.73/ 7.25	10.85± 0.18	31.62	53.51

Table A. Comparison of the performances of CHO-NTS_1 cells transiently transfected with pCAeq plasmid and of the stable AequoScreen CHO-NTS_1 cells. Representative experiment out of 3 independent experiments, reader MicroBeta JET, 96-well.

Ligand	**CCK8 sulfated (agonist)**				**LY225910 (antagonist)**	
	pEC_{50}± SD	S/B	Top/Digit%	Z'/CV	pIC_{50}± SD	Z' / CV
Cholecystokinin CCK_2 AequoScreen cell line	9.74± 0.17	65.27	47.02	0.77/ 6.83	11.58± 0.09	0.65/ 8.22
1321N1 Cholecystokinin CCK_2 cell line transiently transfected with pCAeq	9.86± 0.32	107.97	32.19	0.70 /8.54	10.85± 0.18	0.62/ 7.35

Table B. Comparison of the performance between 1321N1-CCK_2 cells transiently transfected with pCAeq plasmid and the stable AequoScreen 1321N1-CCK_2 cell line. Representative experiment out of 3 independent experiments, reader MicroLumat, 96-well.

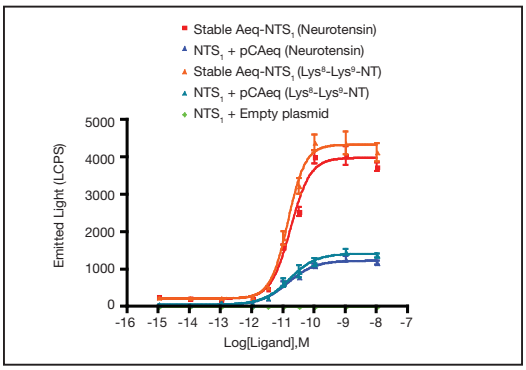


Figure 1. Aequorin response to NTS_1 agonists. The pCAeq AequoScreen plasmid (ES-002-AC) was transiently transfected using Lipofectamine reagent in CHO-NTS_1 cell line (ES-690-C), the aequorin signal was studied 48 hours after the transfection and compared with the stable Neurotensin NTS_1 AequoScreen cell line (ES-690-A). Reader: MicroBeta JET, 96-well.

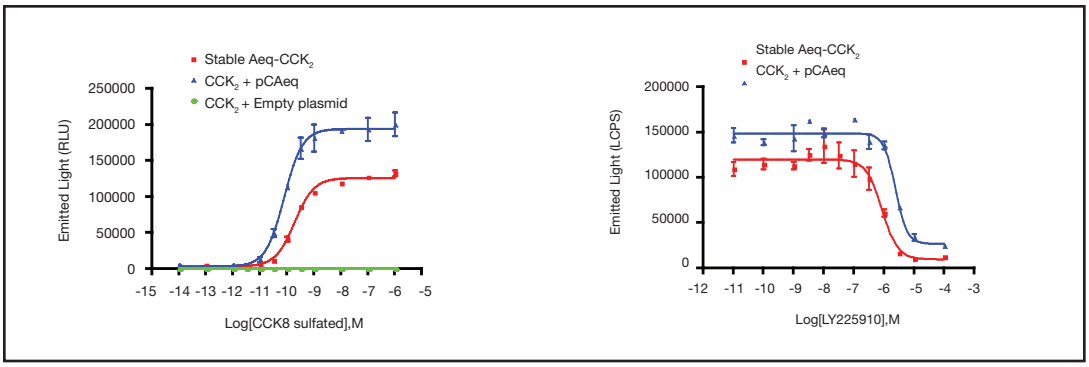


Figure 2. Aequorin response to CCK_2 agonists and antagonists. The pCAeq AequoScreen plasmid (ES-002-AC) was transiently transfected using Lipofectamine reagent in 1321N1 Cholecystokinin CCK_2 cell line (ES-531-C). The activity of the aequorin was studied 48 hours after the transfection and compared with the stable Cholecystokinin CCK_2 AequoScreen cell line (ES-531-A). Reader: MicroLumat, 96-well MicroBeta JET, 96-well.

G_s-coupled receptors

The two model G_s coupled receptors chosen were CHO Histamine H_2 (ES-391-C and ES-391-A) and HEK293 Adenosine A_{2B} (ES-013-C and ES-013-A) cell lines. The summary of these experiments is presented in graphical data in Figures 3, 4 and in Tables C and D.

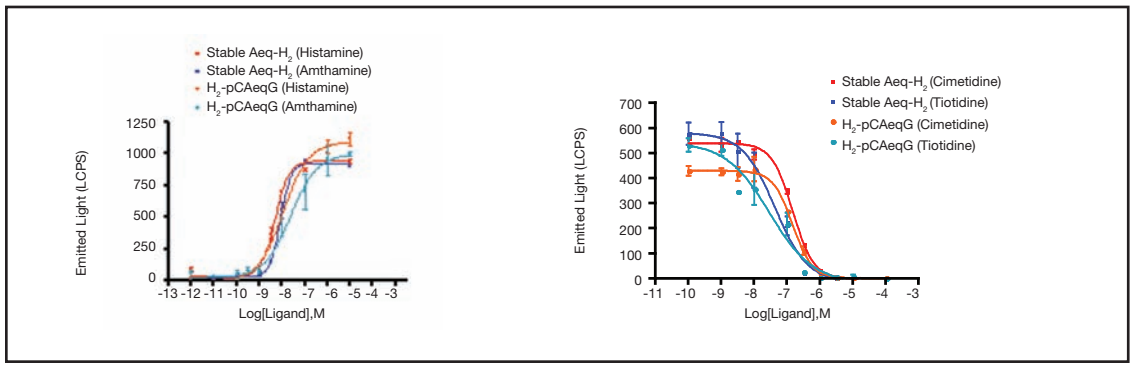


Figure 3. Aequorin response to H_2 agonists and antagonists. The pCAeqG AequoScreen plasmid (ES-003-AC) was transiently transfected by Lipofectamine reagent in CHO Histamine H_2 (human) cell line (ES-391-C), the activity of the aequorin was studied 48 hours after the transfection and compared with the stable Histamine H_2 AequoScreen cell line (ES-391-A). Reader: MicroBeta JET, 96-well.

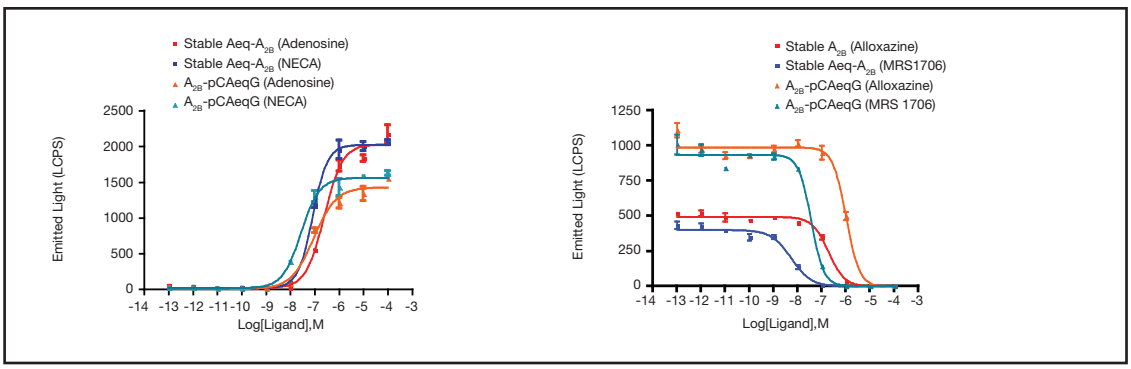


Figure 4. Aequorin response to Adenosine A_{2B} agonists and antagonists. The pCAeqG AequoScreen plasmid (ES-003-AC) was transiently transfected by Lipofectamine reagent in HEK293 Adenosine A_{2B} (human) cell line (ES-013-C), the activity of the aequorin was studied 48 hours after the transfection and compared with the stable Adenosine A_{2B} AequoScreen cell line (ES-013-A) Reader: MicroBeta JET, 96-well.

Agonists	**Histamine**				**Amthamine**		
Histamine H_2 AequoScreen cell line	pEC_{50}± SD	S/B	Top/Digit%	Z'/CV	pEC_{50}± SD	S/B	Top/Digit%
	8.01± 0.26	53.74	109.04	0.64/ 11.34	7.82± 0.28	50.82	102.83
CHO Histamine H_2 cell line transiently transfected with pCAeqG	7.82± 0.28	60.63	58.01	0.68/ 9.51	7.51± 0.15	56.38	54.18

Antagonists	**Cimetidine**		**Tiotidine**
Histamine H_2 AequoScreen cell line	pIC_{50}± SD	Z' / CV	pIC_{50}± SD
	6.85± 0.04	0.81/ 5.77	7.70± 0.13
CHO Histamine H_2 cell line transiently transfected with pCAeqG	6.78± 0.03	0.72/ 8.37	7.68± 0.03

Table C. Comparison of the performance between CHO-H_2 cells transiently transfected with pCAeqG plasmid and the stable AequoScreen CHO-H_2 cells. Representative experiment out of 3 independent experiments, reader MicroBeta JET, 96-well.

Agonists	Adenosine				NECA		
Adenosine A_{2B} AequoScreen cell line	pEC_{50}± SD	S/B	Top/Digit%	Z'/CV	pEC_{50}± SD	S/B	Top/Digit%
	6.37± 0.36	58.84	72,00	0.68 / 9.25	7.05± 0.22	74.96	75.88
HEK293 Adenosine A_{2B} cell line transiently transfected with pCAeqG	6.89± 0.34	53.22	52.75	0.60 / 12.08	7.44± 0.11	53.83	51.78

Antagonists	Alloxazine		MRS 1706
Adenosine A_{2B} AequoScreen cell line	pIC_{50}± SD	Z'/CV	pIC_{50}± SD
	6.69± 0.08	0.64 / 11.06	7.92± 0.29
HEK293 Adenosine A_{2B} cell line transiently transfected with pCAeqG	5.94± 0.20	0.66 / 10.95	7.28± 0.28

Table D. Comparison of the performance between HEK293-A_{2B} cells transiently transfected with pCAeqG AequoScreen plasmid and the stable AequoScreen HEK293-A_{2B} cells. Representative experiment out of 3 independent experiments, reader MicroBeta JET, 96-well.

Ligand	Somatostatin 28 (Agonist)			Somatostatin 14 (Agonist)				CYN154806 (Antagonist)	
CHO Somatostatin sst_2 cell line transiently transfected with pCAeqG	pEC_{50}± SD	S/B	Top/Digit%	pEC_{50}± SD	S/B	Top/Digit%	Z'/CV	pEC_{50}± SD	Z'/CV
	8.23± 0.09	26.62	24.9	8.35± 0.18	27.15	25.4	0.61/11.17	6.19± 0.18	0.84/3.57

Table E. HEK293-A_{2B} cells transiently transfected with pCAeqG plasmid and the stable AequoScreen HEK293-A_{2B} cells. Representative experiment out of 2 independent experiments, reader MicroBeta JET, 96-well.

G_i-coupled receptors

The model G_i coupled receptor chosen was CHO Somatostatin sst_2 cell line. The summary of these experiments is presented in graphical data in Figure 5 and in Table E.

Conclusions

All the data clearly illustrated that transient introduction of an aequorin expression vector allows development of an aequorin assay from an initially non-aequorin cell line. The pharmacological values observed in the agonist and antagonist assays gave EC_{50} and IC_{50} values in the expected range, and signal intensity is compatible with the development of HTS assays using cells transiently transfected with these plasmids.

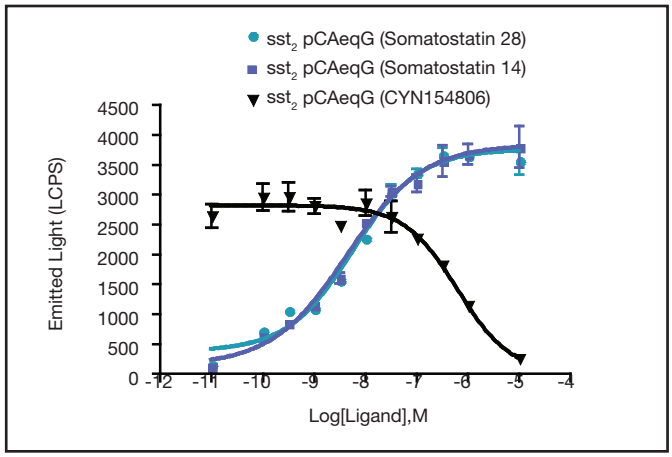


Figure 5. Aequorin response to Somatostatin sst_2 agonists and antagonists. The pCAeqG AequoScreen plasmid (ES-003-AC) was transiently transfected by Lipofectamine reagent in CHO Somatostatin sst_2 (human) cell line, the activity of the aequorin was studied 48 hours after the transfection. Representative experiment out of 3 independent experiments. Reader: MicroBeta JET, 96-well. (for this receptor, no stable cell line was available at the moment of the assay).

Materials

Description		Source	Catalog No.
AequoScreen Plasmid	Plasmid pCAEq	PerkinElmer	ES-002-AC
AequoScreen Plasmid (+$G\alpha_{16}$)	Plasmid pCAeqG	PerkinElmer	ES-003-AC
Neurotensin NTS_1 (human) ValiScreen™ cell line		PerkinElmer	ES-690-C
Histamine H_2 (human) ValiScreen cell line		PerkinElmer	ES-391-C
Cholecystokinin CCK_2 (human) ValiScreen cell line		PerkinElmer	ES-531-C
Adenosine A_{2B} (human) ValiScreen cell line		PerkinElmer	ES-013-C
Neurotensin NTS_1 AequoScreen cell line		PerkinElmer	ES-690-A
Histamine H_2 AequoScreen cell line		PerkinElmer	ES-391-A
Cholecystokinin CCK_2 AequoScreen cell line		PerkinElmer	ES-531-A
Adenosine A_{2B} AequoScreen cell line		PerkinElmer	ES-013-A
Somatostatin sst_{2a} (human) ValiScreen cell line		PerkinElmer	ES-521-C
MicroBeta JET Luminescence and Scintilation Counter		PerkinElmer	1450-221; 1450-222
OptiPlate-96 (96-well white micropate)	50 plates	PerkinElmer	6005290
Amthamine		Tocris	668
ATP		Sigma	A7699
Cimetidine		Tocris	902
Coelenterazine h		Promega	S2011
CYNI54806		Tocris	1843
Digitonin		Sigma	37006
DMEM		Lonza	BE12-604F
DMEM/Ham's F12 (with 15 mM HEPES, L-glutamine, without phenol red)		Invitrogen	11039
DPBS		Lonza	BE17-515Q
EMEM		Lonza	BE06-174G
Fetal Bovine Serum (FBS)		JRH	12103-1000M
G418		Invitrogen	10131-027
Ham's F12 medium (with L-glutamine)		Invitrogen	21765
Histamine		Sigma	H7250
Lipofectamine™ 2000		Invitrogen	11668019
[$Lys^{8®}$ Lys^9]NT (8-13)		Bachem	H8370
Methanol		Merck	K36875909705
Minisorp 75X12		Nunc	443990
Neurotensin		Bachem	H4435
Opti-MEM® I Reduced Serum Medium		Invitrogen	31985062
Pen/Strep		Lonza	DE17-602E
Protease-free BSA		Serva	11926
Puromycin		Sigma	P7255
Sodium pyruvate		Invitrogen	11360-039
Somatostatin 14		Bachem	H1490
Somatostatin 28		Bachem	H4955
SR142948		Tocris	2309
Tiotidine		Tocris	826
Trypsin (0.25g/L)		Lonza	BESP192

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