AequoZen™ frozen cells Starter Kit

Catalog Number:

ES-001-AF

For Laboratory Use Only
Research Chemicals for Research Purposes Only

Precautions

- Upon receipt, store the vials immediately in liquid nitrogen.
- This kit contains living cells. These cells should be prepared as described on page 10.

TABLE OF CONTENTS

Before Starting

A. Kit Content

- 2 vials of H_1 cell line (ES-390-AS)
- 4 vials of M_5 cell line (ES-214-AS)
- Protocol : Provides all the instructions necessary to help you run the AequoScreen assay

B. Receiving the AequoZen frozen cells Starter Kit

Upon receiving the AequoZen frozen cells Starter Kit, ensure that the kit package still contains dry ice and that the ice is not completely evaporated. Verify that all components are present in the kit using the list above. Store the vials immediately in liquid nitrogen.

C. Recommended additional Reagents and Materials

Item	Suggested source	Catalog #
Ham's F12 medium	Invitrogen	21765
DMEM/Ham's F12	Invitrogen	11039
Protease-free BSA	Serva	11926
Coelenterazine h	Promega	S2011
Digitonin	Sigma	37006
ATP	Sigma	A-7699
OptiPlate™-96	PerkinElmer	6005290
Black clear bottomed 384 microplate	Greiner	781096
Black 384 microplate	Greiner	781076
Tips P30	PerkinElmer	6900027
Minisorp 75x12	Nunc	443990
Histamine dihydrochloride	Sigma	H-7250
HTMT (6-[2-(4-Imidazolyl)ethylamino]-n-(4-trifluoromethylphenyl) heptanecarboxamide dimaleate)	Tocris	646
trans-tripolidine hydrochloride	Sigma	T-118
Pyrilamine maleate (Mepyramine maleate)	Tocris	660
Acetylcholine chloride	Sigma	A9101
Oxotremorine (N,N,N,-trimethyl-4-(2-oxo-1-pyridinyl)-2-butyn-1-ammonium iodide)	Tocris	1067
N-Me-Scopolamine ((-) Scopolamine methyl bromide)	Sigma	S8502
Atropine sulphate crystalline	Sigma	A0257

II. Introduction

GPCR screening

G protein coupled receptors (GPCRs) have been considered as a highly "druggable" target for many years, with over 40% of marketed drugs acting to modulate their function. For many years, radiometric techniques have dominated GPCR screening. However in the last decade the development of functional assays, where the effect of molecules is evaluated in terms of GPCR activation, has accelerated.
In particular, measurement of calcium signaling and the development of molecular strategies which couple the majority of GPCRs to calcium signaling has allowed the use of high-throughput functional screening in GPCR research.

Calcium signaling and Aequorin

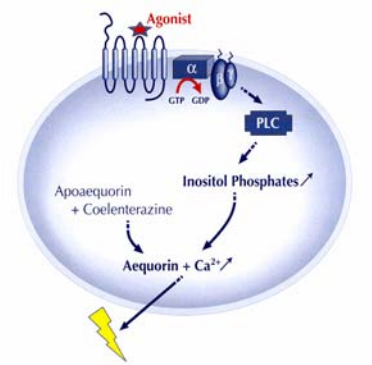
Aequorin is a photoprotein originating from the jellyfish Aequorea Victoria (Proc. Nat. Acad. Sci. USA 82: 3154-3158, 1985; Biochem. Biophys. Res. Commun. 126: 1259-1268, 1985). The apo-enzyme (apoaequorin) is a 21 kD protein that needs a hydrophobic prosthetic group, coelenterazine, to be converted 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 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) (J. Biol. Chem. 270: 9896-9903, 1995; 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 (J. Biol. Chem. 270: 9896-9903, 1995).

Aequorin cell line development

Sheu et al. (Anal. Biochem. 209: 343-347, 1993) and Button & Brownstein (Cell Calcium 14 : 663-671, 1993) first described the use of recombinant cell lines with stable co-expression of apoaequorin and a GPCR as a system to detect activation of the receptor, following addition of an agonist, via the measurement of light emission. A later report by Stables et al. (Anal. Biochem. 252: 115-126, 1997) showed that when apoaequorin is expressed in the mitochondria, the emission of light upon stimulation of a GPCR was higher than when apoaequorin is expressed in the cytoplasm.

Coupling to calcium has been optimized to provide the highest sensitivity of detection.

Stable aequorin parental cell lines were first generated by transfection of wild type CHO-K1 cells with a bicistronic plasmid containing the sequence of the mitochondrially targeted aequorin. The stable parental cell lines were then transfected with a bicistronic vector containing the sequence of the GPCR of interest.



H_1 and M_5 receptors

The human histamine H_1 and muscarinic M_5 receptors belong to the family of G-protein coupled receptors (GPCR).

The histamine H_1 receptor is well-characterized and is an important target associated with allergies. It operates through the inositol phosphate/diacylglycerol second messenger, and is a $G_{q/11}$ class receptor. Among the many responses mediated by the H_1 receptor are smooth muscle contraction, increased vascular permeability, hormone release, and cerebral glyconeogenesis. (Biochem. Soc. Trans. 20: 122-125, 1992). H_1 antagonists are used in the treatment of allergic and anaphylactic reactions as well as various inflammatory conditions (JPET, 288: 858-865, 1999; Pharmazie, 59: 409-411, 2004).

The muscarinic receptors mediate the metabotropic actions of acetylcholine in the nervous system. Little is known about the physiological role of the M_5 receptor. M_5 exhibits an antagonist affinity profile similar to that of M_3 (British Pharmacol. Society, 127: 590-596, 1999; Auton. Autacoid. Pharmacol. 26: 219-233, 2006). This specific subtype of the muscarinic receptor is found in different locations including the salivary glands, substantia nigra and the ventral segmental area of the brain. It is involved in modulating several pharmacological and behavioral functions (Life Sci. 5: 345-353, 2003). The M_5 receptor is also a $G_{q/11}$ receptor, which activates the inositol phosphate/diacylglycerol second messenger.

This kit contains two cell lines with receptors coupled to G_q. The human histamine H_1 cells generate a strong calcium response while the human muscarinic M_5 cell line was selected because it generates a weaker calcium response. This unables you to become familiar with the AequoScreen® technology.

III. AequoZen H_1 Cell Line

RECEPTOR:	**HISTAMINE**
SUBTYPE:	**H_1**
SPECIES:	**human**
Catalog n°:	**ES-390-AS**

Cell line identification:	H_1-A12
Origin:	Stable recombinant CHO-K1 cell line expressing the mitochondrially-targeted Aequorin and the histamine H_1 receptor (GenBank : NM_000861), γ-irradiated
Pack size:	10 x 10^6 cells/ml
Volume per vial:	2.5 ml

Storage conditions:
Upon receipt, store vials immediately in liquid nitrogen

IV. AequoScreen® Assay for H_1

A. Materials

- Culture medium: Ham's F12 medium (Invitrogen) + 10% FBS
- BSA medium: 500 ml DMEM/Ham's F12 (with 15 mM HEPES, L-glutamine, without phenol red) culture medium (Invitrogen) + 5 ml of 10% protease-free BSA in H_2O (final BSA concentration is 0.1 %)
- Coelenterazine h: prepare 500 µM stock solution, resuspend 10mg of Coelenterazine h in 49.08 ml methanol (Promega). Aliquot and store at -20°C in the dark.
- Digitonin: prepare 50 mM stock solution, dissolve 1 g of Digitonin (Sigma) in 16.27 ml of DMSO. Aliquot and store at -20°C.
- ATP: prepare 100mM stock solution, dissolve 1 g of ATP (Sigma) in 18.1 ml of H_2O. Aliquot and store at -20°C
- Protease-free BSA: Serva
- OptiPlate-96: PerkinElmer Inc. (for MicroBeta® JET)
- Black clear bottomed 384 microplate: Greiner (for LumiLux®)
- Black 384 microplate: Greiner (for EnVision™)
- Tips P30: PerkinElmer Inc. (for LumiLux)
- Tube for compound dilution, Minisorp 75x12: Nunc
- Luminometer :

  LumiLux Cellular Screening platform
  MicroBeta JET Microplate scintillation
  Luminescence counter
  EnVision HTS Ultra sensitive Microplate Reader

B. Ligands

- Reference Agonist: Histamine dihydrochloride (Sigma), diluted in H_2O
- Alternative agonist: HTMT (6-[2-(4-Imidazolyl)ethylamino]-n-(4-trifluoromethylphenyl)heptanecarboxamide dimaleate, Tocris), diluted in H_2O
- Antagonists:

  trans-tripolidine hydrochloride (Sigma), diluted in H_2O
  Pyrilamine maleate (Mepyramine maleate, Tocris), diluted in H_2O

C. General assay procedure

- Thaw cells rapidly by placing the vial in a 37°C water bath for 2 minutes.
- Cells are transferred to a 15ml Falcon tube containing 10ml of culture medium
- Cells are centrifuged, counted and resuspended at $1x10^6$ cells/ml in BSA medium in a Falcon tube.
- Add Coelenterazine h at a final concentration of 5 µM in BSA medium.
- The Falcon tube is wrapped in aluminum foil and placed on a rotating wheel (about 45° angle and 7 rpm/min speed). Alternatively, cells can be gently agitated using a magnetic stirrer.
- Cells are incubated for 4 to 18 h at 20°C (temperature should remain below 25°C).
- Dilute cells with BSA medium (assay media) to the desired concentration* and transfer to a beaker wrapped in aluminum foil on a magnetic stirrer. Use a stirring bar with a ring (low speed).
- Incubate the cells for at least 1 hr at room temperature.

* For the **MicroBeta JET**, use a final concentration of $2.5x10^5$ cells/ml. The minimal volume needed is 50 ml ($1.25x10^7$ cells).

* For the **EnVision**, use a final concentration of $2.5x10^5$ cells/ml. The minimal volume needed is 50 ml ($1.25x10^7$ cells).

* For the **LumiLux**, use a final concentration of $1.25x\ 10^5$ cells/ml. The minimal volume needed will depend on the cell stirrer flask size used, as described in the table below.

Lumilux with the single cell tray:

Flask size	Flask Dead Volume	Tray Dead Volume
1000 ml	Not known	32 ml
500 ml	80 ml	32 ml
250 ml	5 ml	32 ml
125 ml	5 ml	32 ml

LumiLux with the assay development quad cell tray:

Flask size	Flask Dead Volume	Tray Dead Volume
1000 ml	Not known	5 ml
500 ml	80 ml	5 ml
250 ml	5 ml	5 ml
125 ml	5 ml	5 ml

For agonist assay

MicroBeta JET: 96-well format
Inject 50 µl, 12500 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.

EnVision: 384-well format
Inject 20 µl, 5000 cells/well of cell suspension into 20 µl of agonist solution (ligand plate) which has been pre-dispensed into a black 384-well white OptiPlate. Measure the light emitted for 20 s.

LumiLux: 384-well format
Inject 20 µl, 2500 cells/well of cell suspension into 20 µl of agonist solution (ligand plate) which has been pre-dispensed into a 384-well black clear bottomed microplate. Measure the light emitted prior to cell addition for 10 s and a further 30 s upon cell injection.
(Dispense height: 2.5 mm above well; dispense speed: 55 µl/s.)

For antagonist assay

MicroBeta JET: 96-well format
Inject 50 µl, 12500 cells/well of cell suspension into 50 µl of antagonist solution (ligand plate) which has been pre-dispensed into a 96-well white OptiPlate. Incubate cells with antagonist for 15 min at room temperature. Inject 50 µl of agonist (3 x EC_{80} final concentration) onto the mix of cells and antagonist and record the light emitted for 20 s.

LumiLux: 384-well format
Inject 20 µl, 2500 cells/well of cell suspension into 20 µl of antagonist solution (ligand plate) which has been pre-dispensed into a 384-well black clear bottomed microplate. Incubate cells with antagonist for 15 min at room temperature. Inject 20 µl of agonist (3 x EC_{80} final concentration) onto the mix of cells and antagonist and record the light emitted for 10 s prior to agonist addition and 30 s following agonist addition. All steps can be performed using the LumiLux liquid handling and software to schedule incubations.
(Dispense height: 2.5 mm above well; dispense speed: 55 µl/s.).

Positive controls

- Digitonin (100 µM final concentration) is used as a positive control for the coelenterazine cell loading.
- ATP (10 µM final concentration) is used as a positive control for the endogenous response within CHO-K1 cells (purinergic P2Y receptor).

Generation of dose-response curves

The emitted light, after integration, is plotted against the concentration of ligand. EC_{50} are determined using a single site model.

Ligand plates

Ligand dilutions are performed using BSA medium, in Minisorp tubes (silicon tubes) which are kept on ice. Prepare the plate just before running the assay.

Typical dilutions for the LumiLux assay are illustrated below.
The same concentration ranges can be used for the other readers but the dispense volumes per well will vary.

All dilutions should be performed using the BSA medium as described on page 10. The word “buffer” in the following tables refers to the BSA medium.

***Note: The dilutions depend on the stock concentration**

Histamine Agonist — Stock (M) 1.00E-01

	Predilution	Dil	Conc. (M)	Total Vol (µl)	Stock Vol (µl)	Buffer Vol (µl)
		100X	1.0E-03	1000	10	990
		Final Vol/Well = 40 µl		Ligand Vol/Well = 20 µl		

[Final] (Log M)	[Final] (µM)	[Work] (nM)	Volume (µl)	Dilution fold	Ligand Volume (µl)	Buffer Volume (µl)	Remaining in tube (µl)
-6.00	1.00E+00	2000.0	10000	500.0	20	9980	9368
-6.50	3.16E-01	632.46	2000	3.16	632	1368	1368
-7.00	1.00E-01	200.00	2000	3.16	632	1368	1368
-7.50	3.16E-02	63.25	2000	3.16	632	1368	1368
-8.00	1.00E-02	20.00	2000	3.16	632	1368	1368
-8.50	3.16E-03	6.32	2000	3.16	632	1368	1368
-9.00	1.00E-03	2.00	2000	3.16	632	1368	1800
-10.00	1.00E-04	0.20	2000	10.00	200	1800	1800
-11.00	1.00E-05	0.02	2000	10.00	200	1800	1800
-12.00	1.00E-06	0.002	2000	10.00	200	1800	

HTMT Agonist — Stock (M) 1.00E-02

	No predilution	Dil	Conc. (M)	Total Vol (µl)	Stock Vol (µl)	Buffer Vol (µl)
		1X				
		Final Vol/well = 40 µl		Ligand Vol/well = 20 µl		

[Final] (Log M)	[Final] (µM)	[Work] (nM)	Volume (µl)	Dilution fold	Ligand Volume (µl)	Buffer Volume (µl)	Remaining in tube (µl)
-3.50	3.16E+02	632455	2000	15.8	126	1874	1368
-4.00	1.00E+02	200000	2000	3.16	632	1368	1368
-4.50	3.16E+01	63245	2000	3.16	632	1368	1368
-5.00	1.00E+01	20000	2000	3.16	632	1368	1368
-5.50	3.16E+00	6324	2000	3.16	632	1368	1368
-6.00	1.00E+00	2000	2000	3.16	632	1368	1368
-6.50	3.16E-01	632	2000	3.16	632	1368	1368
-7.00	1.00E-01	200	2000	3.16	632	1368	1800
-8.00	1.00E-02	20	2000	10.00	200	1800	1800
-9.00	1.00E-03	2	2000	10.00	200	1800	

***trans*-tripolidine** Antagonist — Stock (M) 1.00E-02

	No predilution	Dil	Conc. (M)	Total Vol (µl)	Stock Vol (µl)	Buffer Vol (µl)
		1X				
		Final Vol/well = 60 µl		Ligand Vol/well = 20 µl		

[Final] (Log M)	[Final] (µM)	[Work] (nM)	Volume (µl)	Dilution fold	Ligand Volume (µl)	Buffer Volume (µl)	Remaining in tube (µl)
-5.00	1.00E+01	30000	2000	333.3	6	1994	1800
-6.00	1.00E+00	3000	2000	10.00	200	1800	1800
-7.00	1.00E-01	300	2000	10.00	200	1800	1368
-7.50	3.16E-02	94.9	2000	3.16	632	1368	1368
-8.00	1.00E-02	30	2000	3.16	632	1368	1368
-8.50	3.16E-03	9.49	2000	3.16	632	1368	1368
-9.00	1.00E-03	3.00	2000	3.16	632	1368	1368
-9.50	3.16E-04	0.95	2000	3.16	632	1368	1368
-10.00	1.00E-04	0.30	2000	3.16	632	1368	1800
-11.00	1.00E-05	0.03	2000	10.00	200	1800	

Pyrilamine Antagonist — Stock (M) 1.00E-02

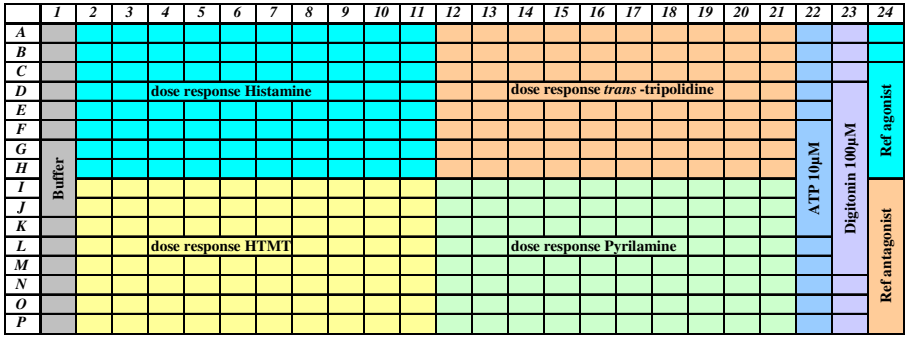
	No predilution	Dil	Conc. (M)	Total Vol (µl)	Stock Vol (µl)	Buffer Vol (µl)
		10X	1.00E-03	100	10	90
		Final Vol/well = 60 µl		Ligand Vol/well = 20 µl		

[Final] (Log M)	[Final] (µM)	[Work] (nM)	Volume (µl)	Dilution fold	Ligand Volume (µl)	Buffer Volume (µl)	Remaining in tube (µl)
-6.00	1.00E+00	3000	3333	333.3	10	3323	3133
-7.00	1.00E-01	300	2000	10.00	200	1800	1800
-8.00	1.00E-02	30	2000	10.00	200	1800	1368
-8.50	3.16E-03	9.49	2000	3.16	632	1368	1368
-9.00	1.00E-03	3.00	2000	3.16	632	1368	1368
-9.50	3.16E-04	0.95	2000	3.16	632	1368	1368
-10.00	1.00E-04	0.30	2000	3.16	632	1368	1800
-11.00	1.00E-05	0.03	2000	10.00	200	1800	1800
-12.00	1.00E-06	0.003	2000	10.00	200	1800	1800
-13.00	1.00E-07	0.0003	2000	10.00	200	1800	

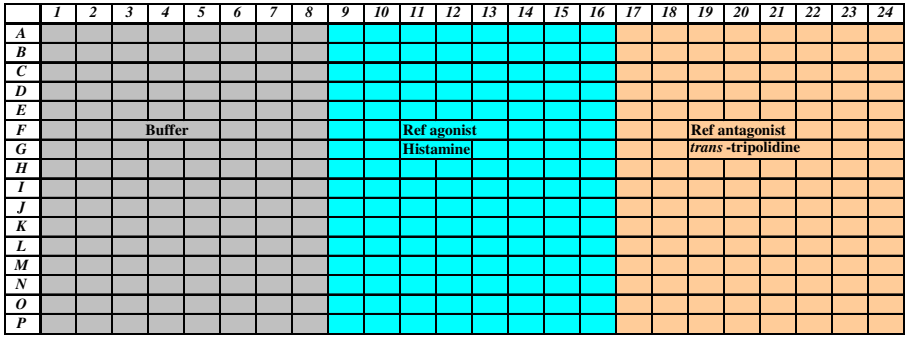
D. Example of ligand plate map

This section contains suggested plate maps for the various assay formats which can be run on the various instruments.

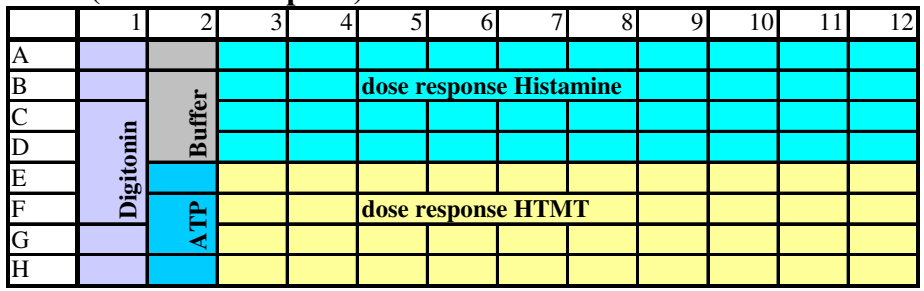
Agonist and antagonist Dose-Response curves on the LumiLux (384-well microplate):



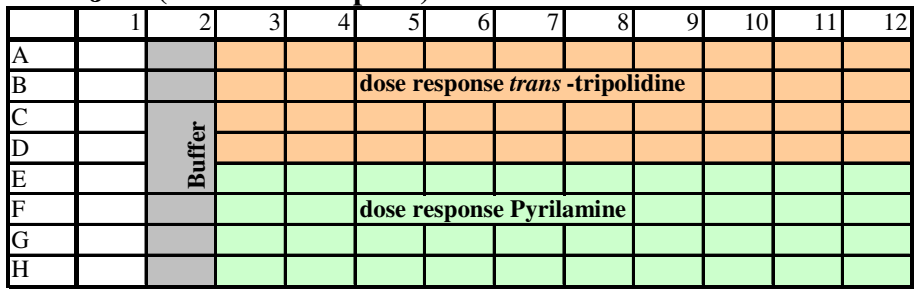
Z' plate on the LumiLux (384-well microplate):



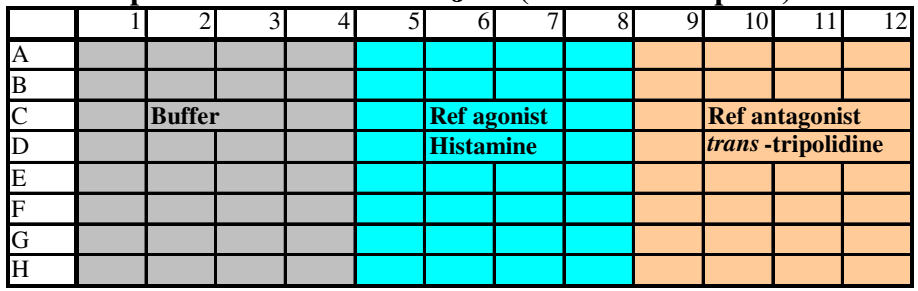
Agonist Dose-Response curves on the MicroBeta JET (96-well microplate):



Antagonist Dose-Response curves on the MicroBeta JET (96-well microplate):



Z' plate on the MicroBeta JET (96-well microplate):



Dose-Response curves and Z' determination on the EnVision (384-well microplate):

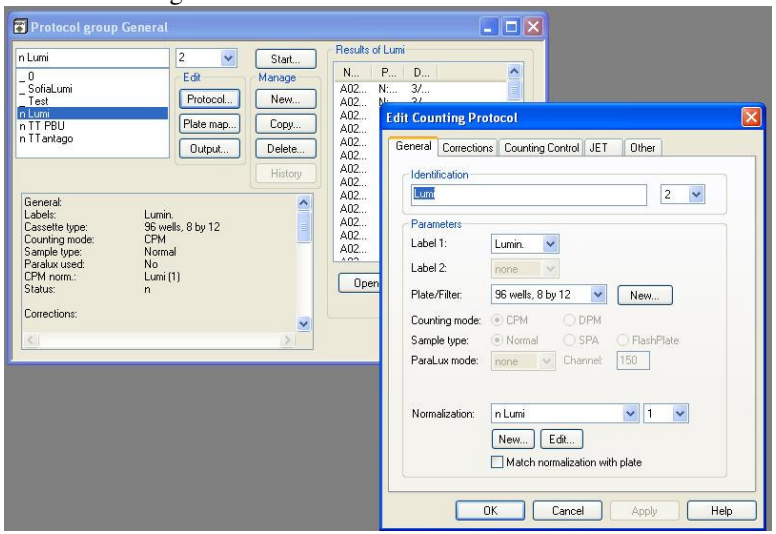
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A																								
B	Buffer			dose response Histamine								ATP 10µM	Digitonin 100µM		Buffer		Ref agonist		Ref antagonist					
C																								
D																								
E				dose response HTMT																				
F																								
G																								
H																								
I																								
J																								
K																								
L																								
M																								
N																								
O																								
P																								

E. Instrument Settings

MicroBeta JET:

(MicroBeta Workstation for Windows V3.0 Release 2)
In Protocols/General → Choose your protocol

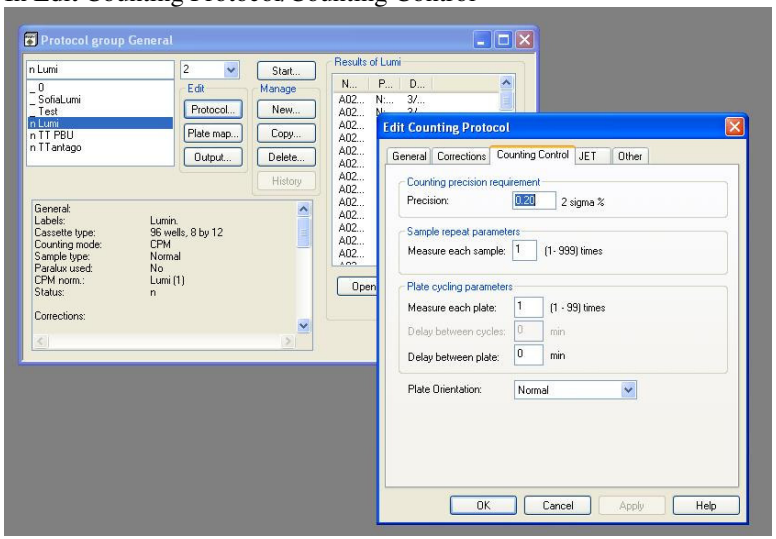
In Edit Counting Protocol/General



- Select Lumin. in label 1
- Select the 96 wells in Plate/Filter

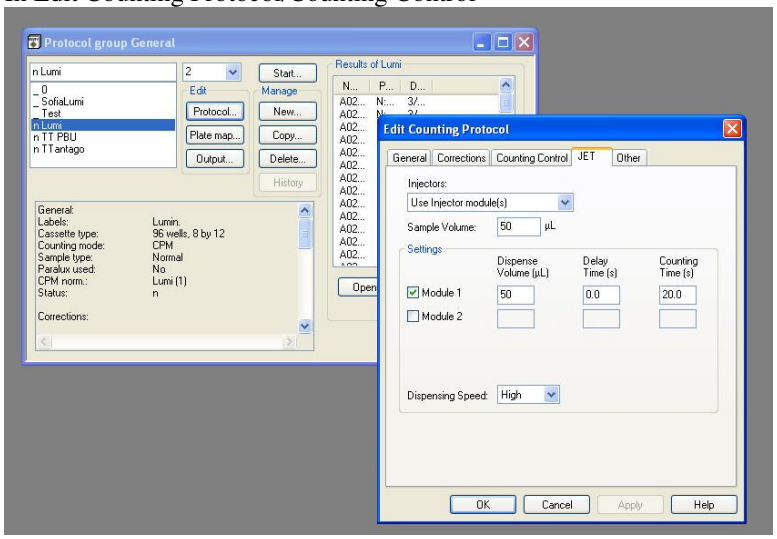
In Edit Counting Protocol/Correction
Click on None for the Background correction

In Edit Counting Protocol/Counting Control



- Select the Precision “0.20”

In Edit Counting Protocol/Counting Control



- Enter “50” for Dispense Volume
- Click on Module 1
- Select High on Dispensing speed

In Injector Setup

- Click on “Initialize the module”

Results

- Open your file
- Results are in “LCPS column” in LCPS unit

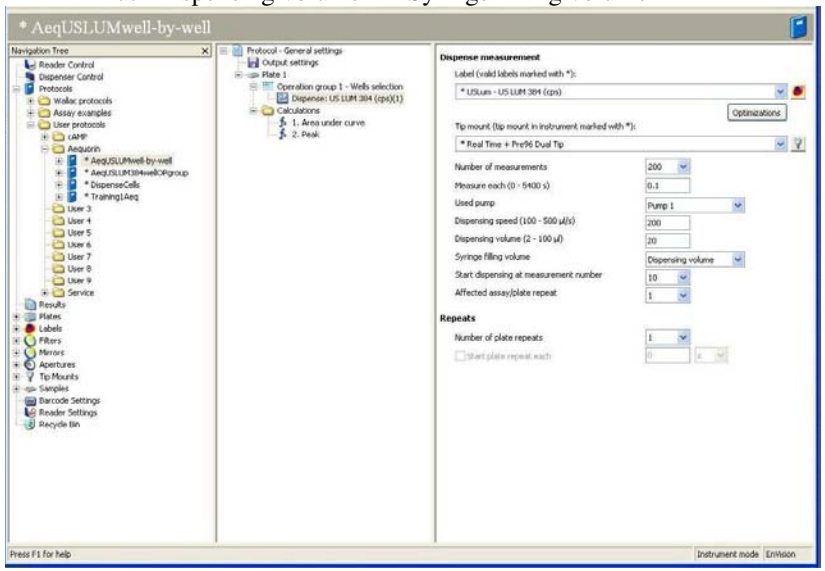
EnVision:

(Wallac EnVision Software version 1.09)
In Protocols/Aequorin

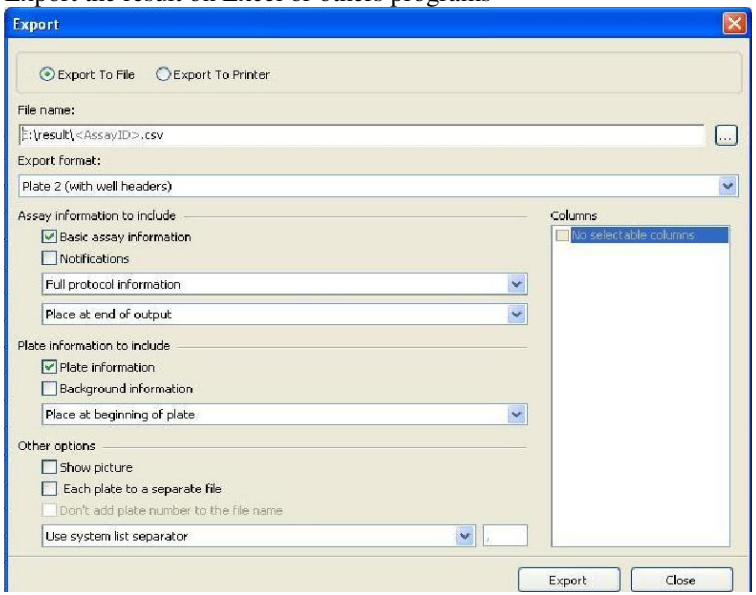
- Select *AeqUS LUMwell-by-well

In dispense:US LUM 384 (cps) (1)

- Enter “200” for the Number of measure
- Enter “0.1” s for Measure each
- Select your pump
- Put “200” in Dispense speed
- Put “20” in Dispense volume
- Put “Dispensing volume” in Syringe filling volume



Export the result on Excel or others programs



Results

- Results are in “Area under curve column” in CPS unit

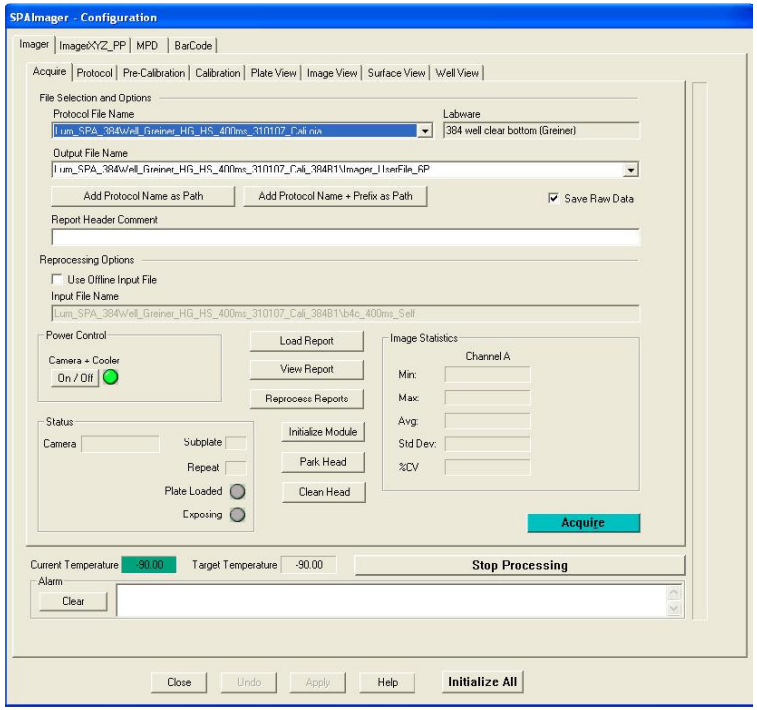
LumiLux:

(AssayPro™ Server for CellLux and LumiLux version 2.2)
Open

- Win Prep for LumiLux
- AssayPro for analysis

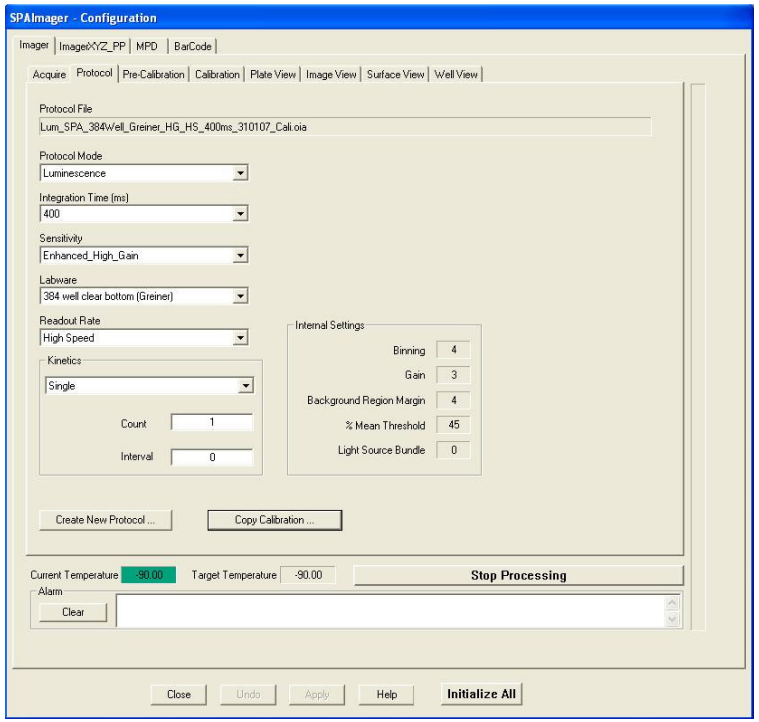
In Utilities/Setup/SPA imager configuration
In Imager/Acquire

- Verify temperature of the camera, -90.00
- Choose your Protocol File Name



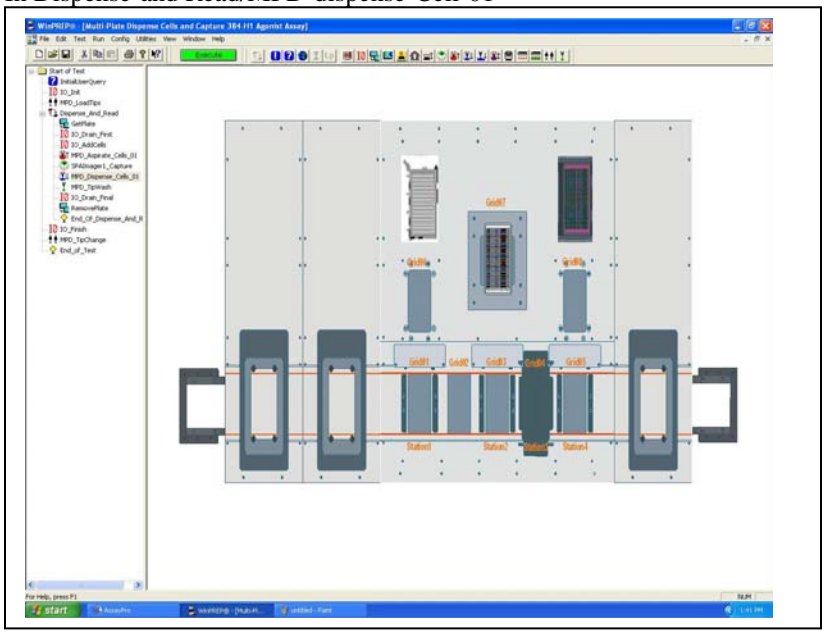
In Imager/Protocol

- Select Luminescence in Protocol Mode
- Select Enhanced_High_Gain in Sensitivity



- Verify the LumiLux Checklist (Provided with the LumiLux Manual)

In Dispense-and Read/MPD-dispense-Cell-01



- Select the name of the plate in Labware

Protocol Summary:

	Cells Injection	Agonist Injection (in Antagonist Assay)
Aspirate height (mm)	2	2
Dispense height (mm)	2.5	2
Dispense volume (µl)	20	20
Dispense speed (µl/s)	55	30

Results

- To run the results with AssayPro
- Results are in “Mean of Resp Area column” in RLU unit

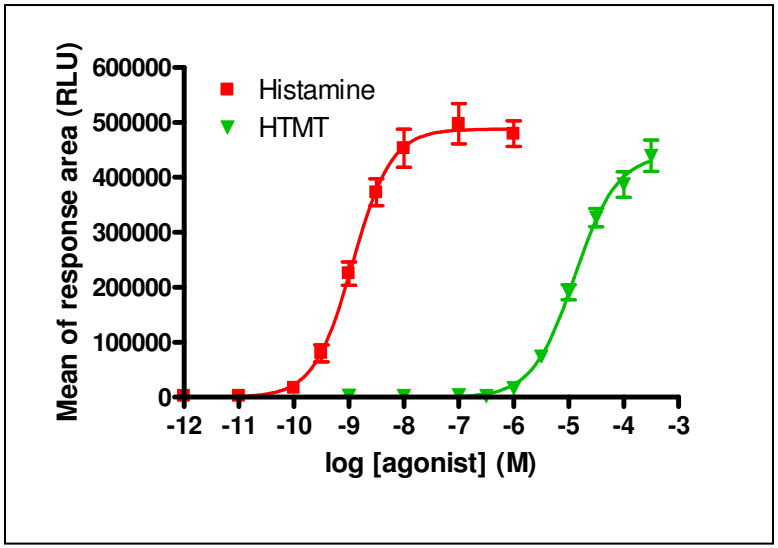
For more information, please contact PerkinElmer technical support

V. Typical Results for H_1

This section contains typical results obtained using the various instruments. Please note that relative light intensity will vary depending on the cell confluency, loading efficiency, cell stress, temperature, etc. These results are shown as a guide only.

A. Agonist Responses on the LumiLux

CHO-H_1 agonist assay, 384-well suspension (2,500 cells/well)

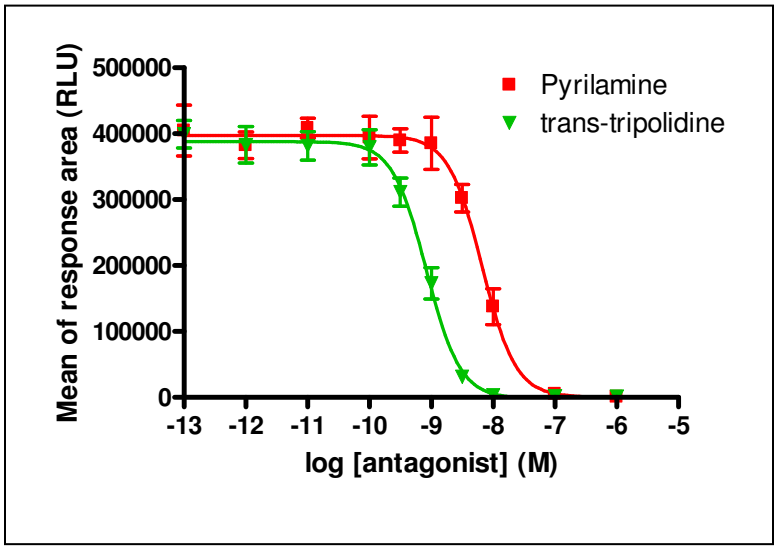


Agonists	pEC_{50} (M)	Digitonin (RLU)	Signal: Background	% Digitonin Response
Histamine	7.92	435095.4	187.26	112.8
HTMT	4.88	435095.4	168.36	101.4

Z' Histamine	0.73	CV	6.42%

B. Antagonist Responses on the LumiLux

CHO-H_1 antagonist assay, 384-well suspension (2,500 cells/well)



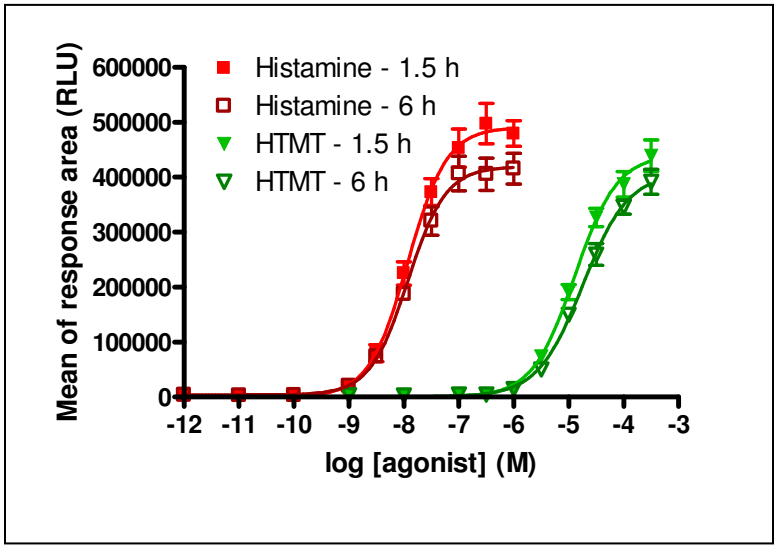
Antagonists	pIC_{50} (M)
trans-tripolidine	9.10
Pyrilamine	8.22

Z' *trans*-tripolidine	0.84

C. Stability of agonist response on the LumiLux

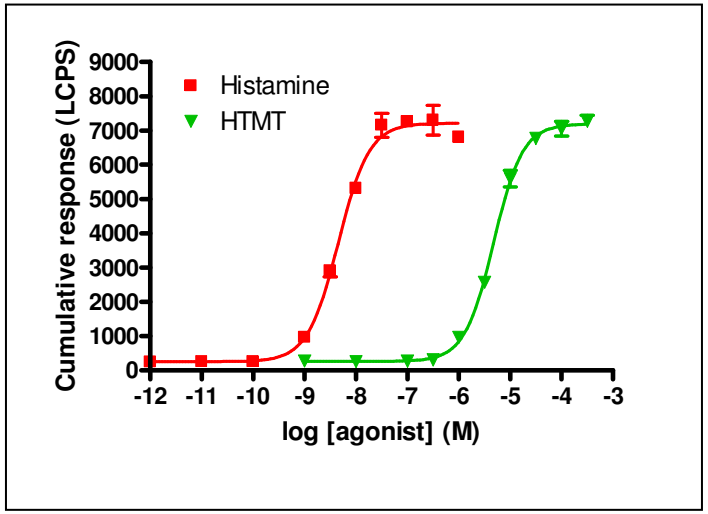
When performing compound screening or other large experiments, a large number of cells are prepared and used over a longer time period. The following graph shows typical agonist results, 1 and 6 hours post-loading while keeping cells in the LumiLux cell stirrer.

CHO-H_1 agonist assay, 1 hour 30 minutes and 6 hours post loading, 384-well suspension (2,500 cells/well)



D. Agonist Response on the MicroBeta JET

CHO-H_1 agonist assay, 96-well suspension (12,500 cells/well).

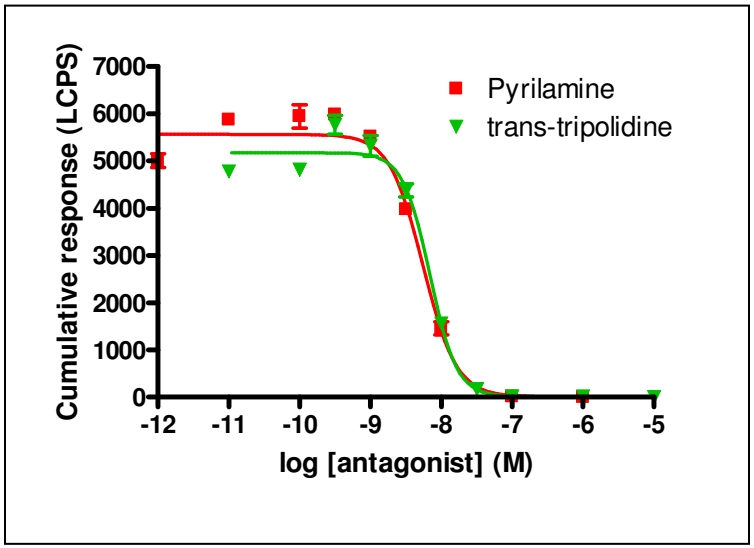


Agonists	pEC_{50} (M)	Digitonin (LCPS)	Signal: Background	% Digitonin Response
Histamine	8.34	5262.90	29.23	137.1
HTMT	5.32	5262.90	29.15	136.7

Z' Histamine	0.72	CV	8.3%

E. Antagonist Response on the MicroBeta JET

CHO-H_1 antagonist assay, 96-well suspension (12,500 cells/well).



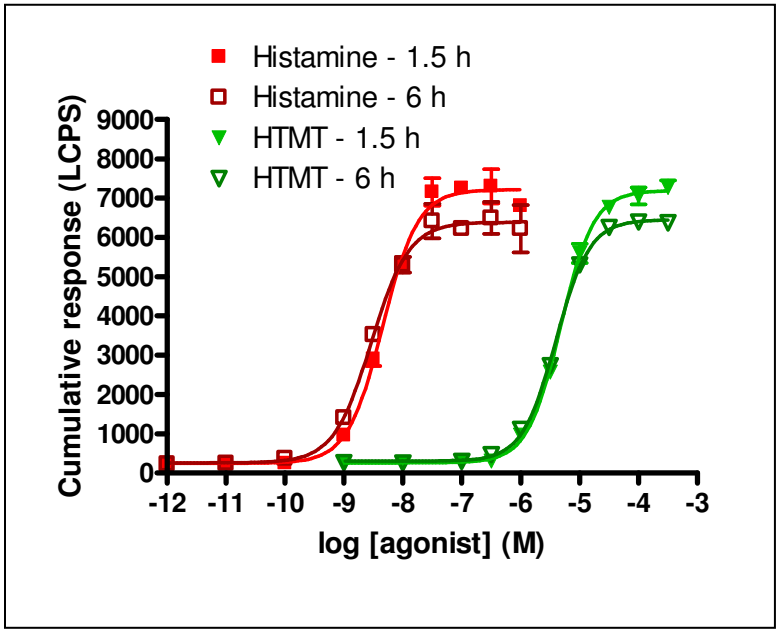
Antagonists	pIC_{50} (M)
trans-tripolidine	8.31
Pyrilamine	8.55

Z' *trans*-tripolidine	0.79

F. Stability of agonist response on the MicroBeta JET

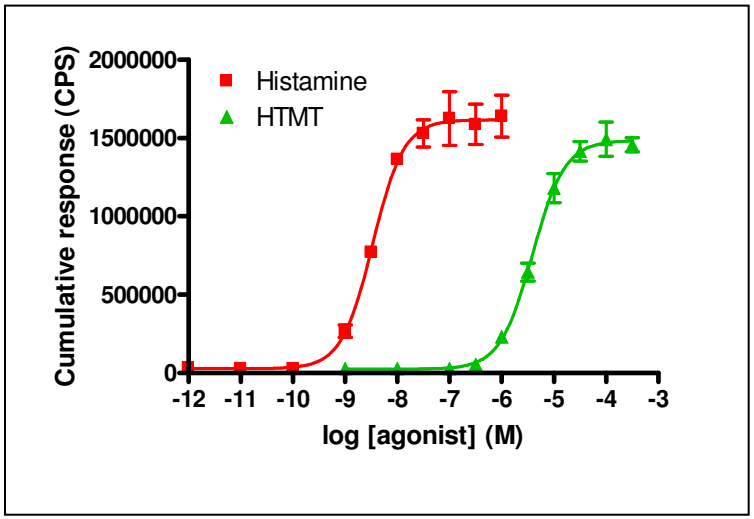
When performing compound screening or other large experiments, a large number of cells are prepared and used over a longer time period. The following graph shows typical agonist results, 1 and 6 hours post-loading.

CHO-H_1 agonist assay, 1 hour 30 minutes and 6 hours post loading, 96-well suspension (12,500 cells/well)



G. Agonist Response on the EnVision

CHO-H_1 agonist assay, 384-well suspension (5,000 cells/well)



Agonists	pEC_{50} (M)	Digitonin (CPS)	Signal: Background	% Digitonin Response
Histamine	8.47	1464076.0	56.40	110.5
HTMT	5.41	1464076.0	51.55	101.4

Z' Histamine	0.84	CV	4.83%

VI. AequoZen M_5 Cell Line

RECEPTOR:	**MUSCARINIC**
SUBTYPE:	**M_5**
SPECIES:	**human**
Catalog n°:	**ES-214-AS**

Cell line identification: M_5-A5

Origin: Stable recombinant CHO-K1 cell line expressing the mitochondrially-targeted Aequorin and the muscarinic M_5 receptor (GenBank : M80333), γ-irradiated

Pack size: 10 x 10^6 cells/ml

Volume per vial: 2.5 ml

Storage conditions:
Upon receipt, store vials immediately in liquid nitrogen

VII. The AequoScreen Assay for M_5

A. Materials

- Culture medium: Ham's F12 medium (Invitrogen) + 10% FBS
- BSA medium: 500 ml DMEM/Ham's F12 (with 15 mM HEPES, L-glutamine, without phenol red) culture medium (Invitrogen) + 5 ml of 10% protease-free BSA in H_2O (final BSA concentration is 0.1 %)
- Coelenterazine h: prepare a 500 µM stock solution, resuspend 10mg of Coelenterazine h in 49.08 ml methanol (Promega). Aliquot and store at -20°C in the dark.
- Digitonin: prepare a 50 mM stock solution, dissolve 1 g of Digitonin (Sigma) in 16.27 ml of DMSO. Aliquot and store at -20°C.
- ATP: prepare a 100mM stock solution, dissolve 1 g of ATP (Sigma) in 18.1 ml of H_2O. Aliquot and store at -20°C
- Protease-free BSA: Serva
- OptiPlate-96: PerkinElmer Inc. (for MicroBeta® JET)
- Black clear bottomed 384 microplate: Greiner (for LumiLux®)
- Black 384 microplate: Greiner (for EnVision™)
- Tips P30: PerkinElmer Inc. (for LumiLux)
- Tube for compound dilution, Minisorp 75x12: Nunc
- Luminometer :

  LumiLux Cellular Screening platform
  MicroBeta JET Microplate scintillation Luminescence counter
  EnVision HTS Ultra sensitive Microplate Reader

B. Ligands

- Reference Agonist: Acetylcholine chloride (Sigma-Aldrich), diluted in H_2O
- Alternative Agonist: Oxotremorine (N,N,N,-trimethyl-4-(2-oxo-1-pyridinyl)-2-butyn-1-ammonium iodide, Tocris), diluted in H_2O
- Antagonists:

  N-Me-Scopolamine ((-) Scopolamine methyl bromide, Sigma-Aldrich), diluted in H_2O

  Atropine (atropine sulphate crystalline, Sigma-Aldrich), diluted in H_2O

C. General assay procedure

- Thaw cells rapidly by placing the vial in a 37°C water bath for 2 minutes.
- Cells are transferred to a 15ml Falcon tube containing 10ml of culture medium.
- Cells are centrifuged, counted and resuspended at $1x10^6$ cells/ml in BSA medium in a Falcon tube.
- Add Coelenterazine h at a final concentration of 5 µM in BSA medium.
- The Falcon tube is wrapped in aluminum foil and placed on a rotating wheel (about 45° angle and 7 rpm/min speed). Alternatively, cells can be gently agitated using a magnetic stirrer.
- Cells are incubated for 4 to 18 h at 20°C (temperature should remain below 25°C).
- Dilute cells with BSA medium (assay media) to the desired concentration* and transfer to a beaker wrapped in aluminum foil on a magnetic stirrer. Use a stirring bar with a ring (low speed).
- Incubate the cells for at least 1 hr at room temperature.

* For the **MicroBeta JET**, use a final concentration of $5x10^5$ cells/ml. The minimal volume needed is 50 ml ($2.5x10^7$ cells).

* For the **EnVision**, use a final concentration of $5x10^5$ cells/ml. The minimal volume needed is 50 ml ($2.5x10^7$ cells).

* For the **LumiLux**, use a final concentration of $2.5x\ 10^5$ cells/ml. The minimal volume needed will depend on the cell stirrer flask size used, as described in the table below.

LumiLux with the single cell tray:

Flask size	Flask Dead Volume	Tray Dead Volume
1000 ml	Not known	32 ml
500 ml	80 ml	32 ml
250 ml	5 ml	32 ml
125 ml	5 ml	32 ml

LumiLux with the assay development quad cell tray:

Flask size	Flask Dead Volume	Tray Dead Volume
1000 ml	Not known	5 ml
500 ml	80 ml	5 ml
250 ml	5 ml	5 ml
125 ml	5 ml	5 ml

For agonist assay

MicroBeta JET: 96-well format

Inject 50 µl, 25000 cells/well of cell suspension into 50 µl of agonist solution (ligand plate) which has been pre-dispensed into a white 96-well Optitplate. Measure the light emitted for 20 s.

EnVision: 384-well format

Inject 20 µl, 10000 cells/well of cell suspension into 20 µl of agonist solution (ligand plate) which has been pre-dispensed into a black 384-well OptiPlate. Measure the light emitted for 20 s.

LumiLux: 384-well format

Inject 20 µl, 5000 cells/well of cell suspension into 20 µl of agonist solution (ligand plate) which has been pre-dispensed into a 384-well black clear bottomed microplate. Measure the light emitted prior to cell addition for 10 s and a further 40 s upon cell injection.
(Dispense height: 2.5 mm above well; dispense speed: 55 µl/s.)

For antagonist assay

MicroBeta JET: 96-well format

Inject 50 µl, 25000 cells/well of cell suspension to 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. Inject 50 µl of agonist (3 x EC_{80} final concentration) onto the mix of cells and antagonist and record the light emitted for 20 s..

LumiLux: 384-well format

Inject 20 µl, 5000 cells/well of cell suspension into 20 µl of antagonist solution (ligand plate) which has been pre-dispensed into a 384-well black clear bottomed microplate. Incubate cells with antagonist for 15 min at room temperature. Inject 20 µl of agonist (3 x EC_{80} final concentration) onto the mix of cells and antagonist and record the light emitted for 10 s prior to agonist addition and 40 s following agonist addition. All steps can be performed using the LumiLux liquid handling and software to schedule incubations.
(Dispense height: 2.5 mm above well; dispense speed: 55 µl/s.)

Positive controls

- Digitonin (100 µM final concentration) is used as a positive control for the coelenterazine cell loading.
- ATP (10 µM final concentration) is used as a positive control for the endogenous response within CHO-K1 cells (purinergic P2Y receptor).

Generation of dose-response curves

The emitted light, after integration, is plotted against the concentration of ligand. EC_{50} are determined using a single site model.

Ligand plates

Ligand(s) dilutions are performed using BSA medium, in Minisorp tubes (silicon tube) which are kept on ice. Prepare the plate just before running the assay.

Typical dilutions for the LumiLux assay are illustrated below.
The same concentration ranges can be used for the other readers but the dispense volumes per well will vary.

All dilutions should be performed using the BSA medium as described on page 34. The word “buffer” in the following tables refers to the BSA medium.

*** Note: The dilutions depend on the stock concentration**

Acetylcholine		Agonist			Stock (M) 1.00E-01		
	Predilution	Dil	Conc. (M)	Total Vol (µl)	Stock Vol (µl)	Buffer Vol (µl)	
		10X	1.00E-02	100	10	90	
		Final Vol/well = 40 µl		Ligand Vol/well = 20 µl			
[Final] (Log M)	[Final] (µM)	[Work] (nM)	Volume (µl)	Dilution fold	Ligand Volume (µl)	Buffer Volume (µl)	Remaining in tube (µl)
-5.00	1.00E+01	20000	10000	500.0	20	9980	9800
-6.00	1.00E+00	2000	2000	10.00	200	1800	1368
-6.50	3.16E-01	632.5	2000	3.16	632	1368	1368
-7.00	1.00E-01	200.0	2000	3.16	632	1368	1368
-7.50	3.16E-02	63.25	2000	3.16	632	1368	1368
-8.00	1.00E-02	20.00	2000	3.16	632	1368	1368
-8.50	3.16E-03	6.32	2000	3.16	632	1368	1368
-9.00	1.00E-03	2.00	2000	3.16	632	1368	1800
-10.00	1.00E-04	0.20	2000	10.00	200	1800	1800
-11.00	1.00E-05	0.02	2000	10.00	200	1800	

Oxotremorine		Agonist			Stock (M) 1.00E-01		
	Predilution	Dil	Conc. (M)	Total Vol (µl)	Stock Vol (µl)	Buffer Vol (µl)	
		10X	1.00E-02	100	10	90	
		Final Vol/well = 40 µl		Ligand Vol/well = 20 µl			
[Final] (Log M)	[Final] (µM)	[Work] (nM)	Volume (µl)	Dilution fold	Ligand Volume (µl)	Buffer Volume (µl)	Remaining in tube (µl)
-5.00	1.00E+01	20000	2000	500.0	4	1996	1800
-6.00	1.00E+00	2000	2000	10.00	200	1800	1368
-6.50	3.16E-01	632.4	2000	3.16	632	1368	1368
-7.00	1.00E-01	200.0	2000	3.16	632	1368	1368
-7.50	3.16E-02	63.24	2000	3.16	632	1368	1368
-8.00	1.00E-02	20.00	2000	3.16	632	1368	1368
-8.50	3.16E-03	6.32	2000	3.16	632	1368	1368
-9.00	1.00E-03	2.00	2000	3.16	632	1368	1800
-10.00	1.00E-04	0.20	2000	10.00	200	1800	1800
-11.00	1.00E-05	0.02	2000	10.00	200	1800	

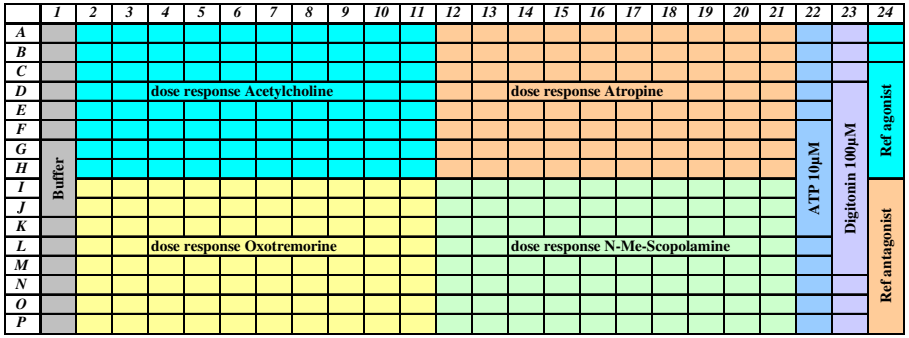
Atropine		Antagonist			Stock (M) 1.00E-02		
	Predilution	Dil	Conc. (M)	Total Vol (µl)	Stock Vol (µl)	Buffer Vol (µl)	
		100X	0.0001	1000	10	990	
		Final Vol/well = 60 µl		Ligand Vol/well = 20 µl			
[Final] (Log M)	[Final] (µM)	[Work] (nM)	Volume (µl)	Dilution fold	Ligand Volume (µl)	Buffer Volume (µl)	Remaining in tube (µl)
-6.00	1.00E+00	3000	2000	33.3	60	1940	1800
-7.00	1.00E-01	300.0	2000	10.00	200	1800	1368
-7.50	3.16E-02	94.86	2000	3.16	632	1368	1368
-8.00	1.00E-02	30.00	2000	3.16	632	1368	1368
-8.50	3.16E-03	9.49	2000	3.16	632	1368	1368
-9.00	1.00E-03	3.00	2000	3.16	632	1368	1368
-9.50	3.16E-04	0.95	2000	3.16	632	1368	1368
-10.00	1.00E-04	0.30	2000	3.16	632	1368	1800
-11.00	1.00E-05	0.03	2000	10.00	200	1800	1800
-12.00	1.00E-06	0.003	2000	10.00	200	1800	

N-Me-Scopolamine		Antagonist			Stock (M) 1.0E-02		
	Predilution	Dil	Conc. (M)	Total vol (µl)	Stock Vol (µl)	Buffer Vol (µl)	
		10X	1.00E-03	100	10	90	
		Final Vol/well = 60 µl		Ligand Vol/well = 20 µl			
[Final] (Log M)	[Final] (µM)	[Work] (nM)	Volume (µl)	Dilution fold	Ligand Volume (µl)	Buffer Volume (µl)	Remaining in tube (µl)
-6.00	1.00E+00	3000.0	2000	333.3	6	1994	1800
-7.00	1.00E-01	300.0	2000	10.00	200	1800	1368
-7.50	3.16E-02	94.9	2000	3.16	632	1368	1368
-8.00	1.00E-02	30.0	2000	3.16	632	1368	1368
-8.50	3.16E-03	9.49	2000	3.16	632	1368	1368
-9.00	1.00E-03	3.00	2000	3.16	632	1368	1368
-9.50	3.16E-04	0.95	2000	3.16	632	1368	1368
-10.00	1.00E-04	0.30	2000	3.16	632	1368	1800
-11.00	1.00E-05	0.03	2000	10.00	200	1800	1800
-12.00	1.00E-06	0.003	2000	10.00	200	1800	

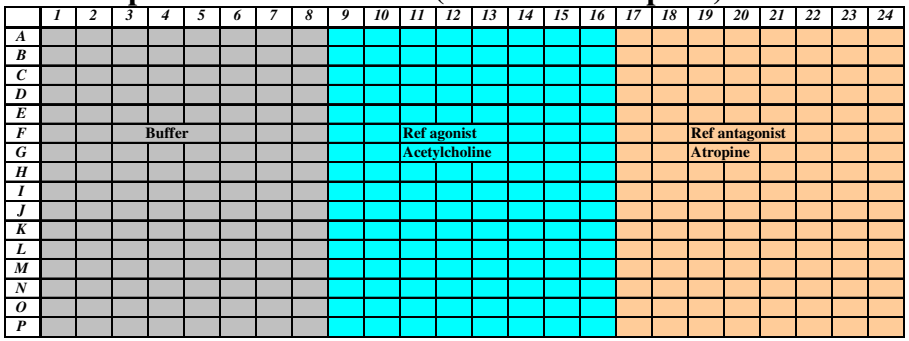
D. Example of ligand plate map

This section contains suggested plate maps for the various assay formats which can be run on the various instruments.

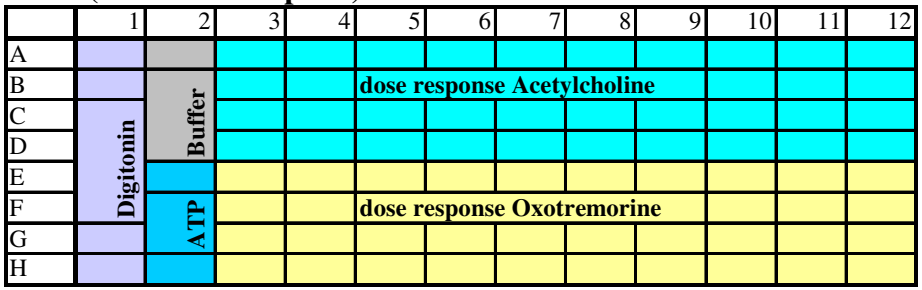
Agonist and antagonist Dose-Response curves on the LumiLux (384-well microplate):



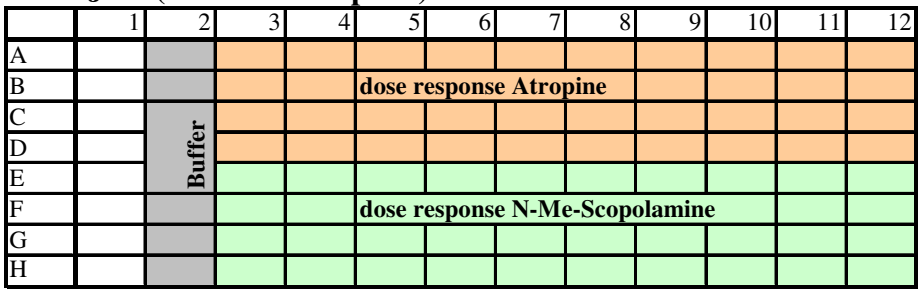
Z' plate on the LumiLux (384-well microplate):



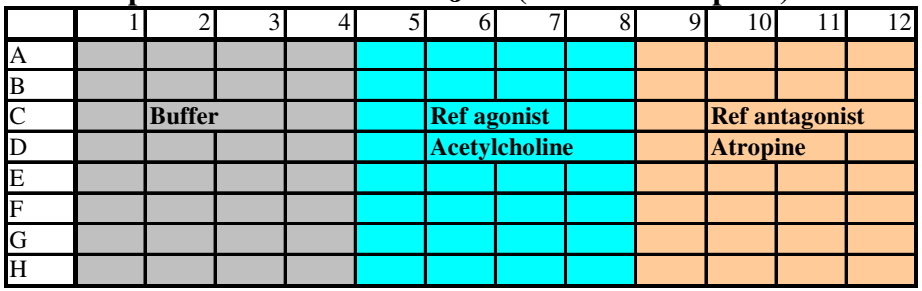
Agonist Dose response curves on the MicroBeta JET (96-well microplate):



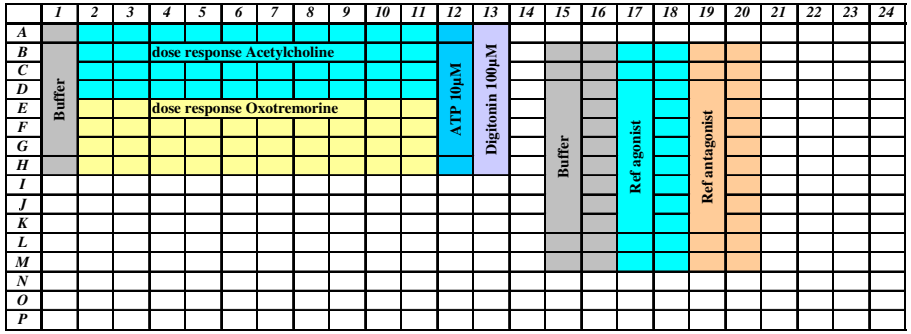
Antagonist Dose-Response curves on the MicroBeta JET (96-well microplate):



Z' plate on the MicroBeta JET (96-well microplate):



Dose-Response curves and Z' determination on the EnVision (384-well microplate):



E. Instrument Settings

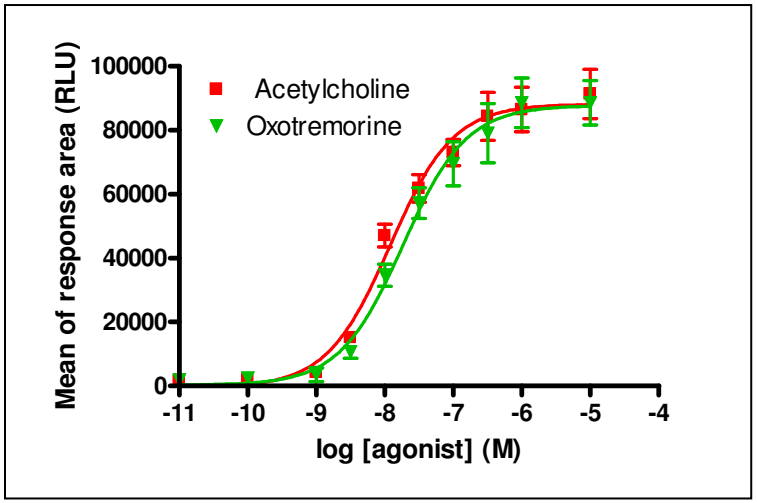
See section IV-E. Instrument Settings for Histamine H_1

VIII. Typical Results for M_5

This section contains typical results obtained using the various instruments. Please note that relative light intensity will vary depending on the cell confluency, loading efficiency, cell stress, temperature, etc. These results are shown as a guide only.

A. Agonist Response on the LumiLux

CHO-M_5 agonist assay, 384-well suspension (5,000 cells/well)

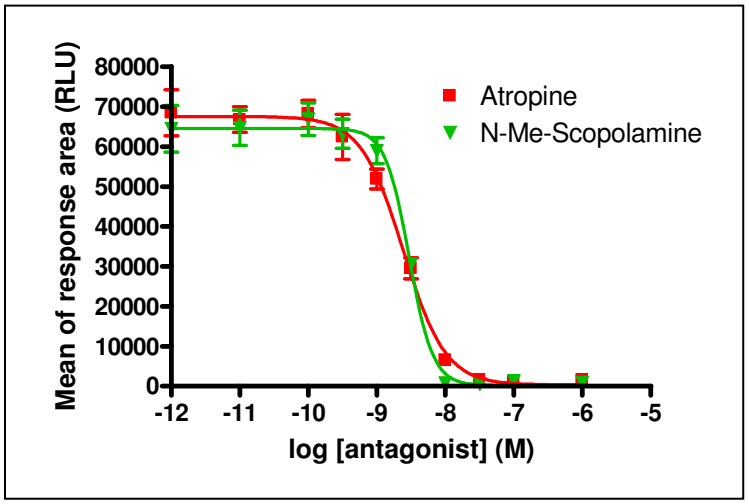


Agonists	pEC_{50} (M)	Digitonin (RLU)	Signal: Background	% Digitonin Response
Acetylcholine	7.92	90747.81	74.81	97.16
Oxotremorine	7.73	90747.81	74.42	96.6

Z' Acetylcholine	0.65	CV	10.25%

B. Antagonist Response on the LumiLux

CHO-M_5 antagonist assay, 384-well suspension (5,000 cells/well)



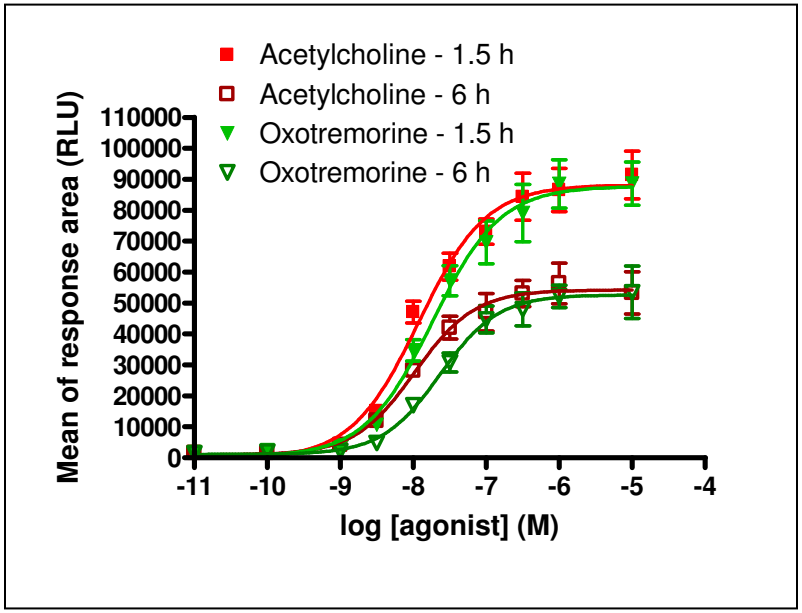
Antagonists	pIC_{50} (M)
Atropine	8.61
N-Me-Scopolamine	8.53

Z' Atropine	0.69

C. Stability of agonist response on the LumiLux

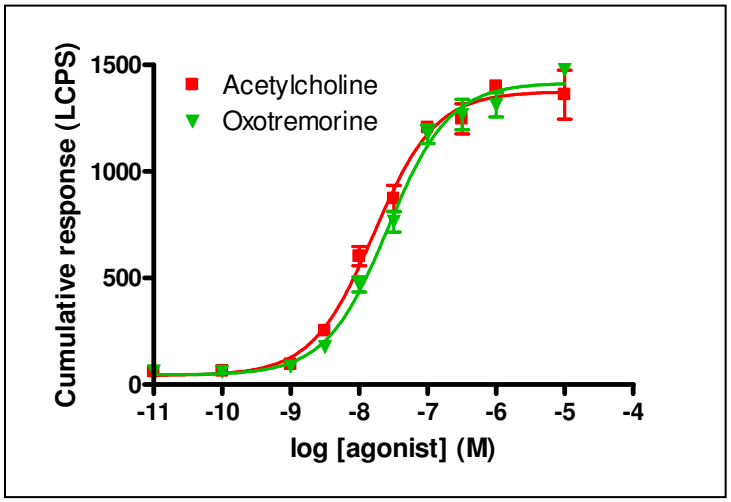
When performing compound screening or other large experiments, a large number of cells are prepared and used over a longer time period. The following graph shows typical agonist results, 1 and 6 hours post-loading while keeping cells in the LumiLux cell stirrer.

CHO-M_5 agonist assay, 1 hour 30 minutes and 6 hours post loading, 384 well suspension (5,000 cells/well).



D. Agonist Response on the MicroBeta JET

CHO-M_5 agonist assay, 96-well suspension (25,000 cells/well).

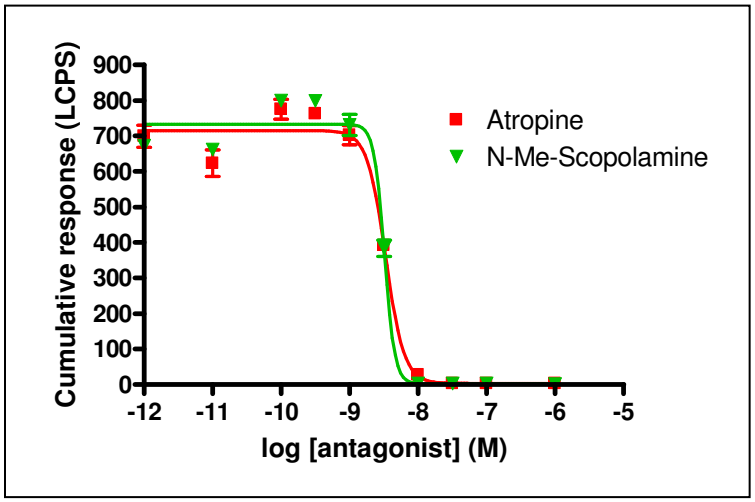


Agonists	pEC_{50} (M)	Digitonin (LCPS)	Signal: Background	% Digitonin Response
Acetylcholine	7.79	1415.31	22.84	97.1
Oxotremorine	7.59	1415.31	23.52	100.0

Z' Acetylcholine	0.64	CV	9.94%

E. Antagonist Response on the MicroBeta JET

CHO-M_5 antagonist assay, 96-well suspension (25,000 cells/well).



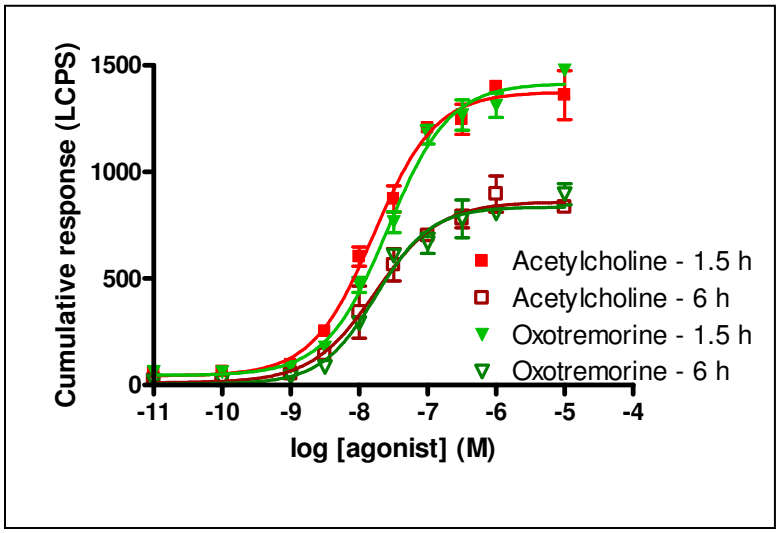
Antagonists	pIC_{50} (M)
Atropine	8.48
N-Me-Scopolamine	8.49

Z' Atropine	0.89

F. Stability of agonist response on the MicroBeta JET

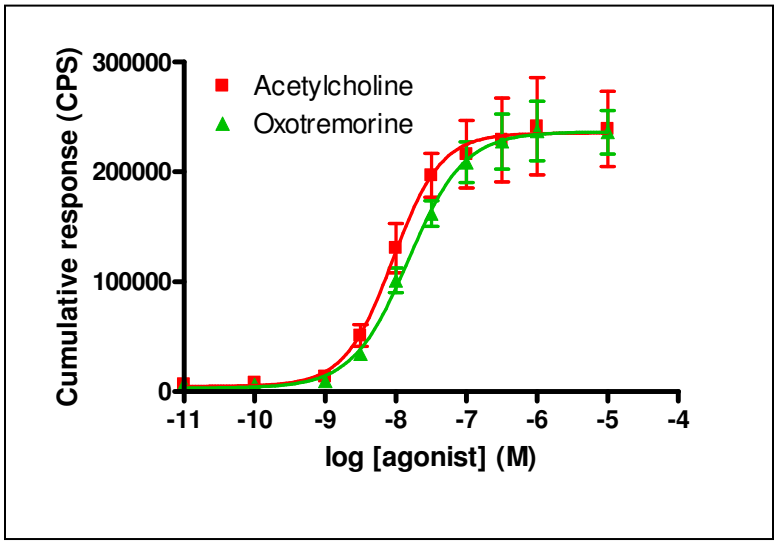
When performing compound screening or other large experiments, a large number of cells are prepared and used over a longer time period. The following graph shows typical agonist results, 1 and 6 hours post-loading.

CHO-M_5 agonist assay, 1 hour 30 minutes and 6 hours post loading, 96 well suspension (25,000 cells/well).



G. Agonist Response on the EnVision

CHO-M_5 agonist assay, 384-well suspension (10,000 cells/well).



Agonists	pEC_{50} (M)	Digitonin (CPS)	Signal: Background	% Digitonin Response
Acetylcholine	8.04	315841.8	36.66	74.5
Oxotremorine	7.82	315841.8	36.82	74.8

Z' Acetylcholine	0.73	CV	8.06%

IX. Troubleshooting Guide

This section describes possible problem that may be encountered with the AequoZen Starter Kit and proposes simple solutions. If more information is required, please contact PerkinElmer Customer Care (see last page for email addresses and phone numbers)

Issue	Possible cause
Cells are not growing	• This is normal. Cells were γ-irradiated.
Cells are not generating any luminescence signal	• Make sure cells were included in the assay. • Check cell viability. • Use fresh coelenterazine and make sure cells were loaded with coelenterazine. • Aequorin generates a flash response: do not wait before reading the well.
There is no agonist response, but a digitonin response	• Make sure the correct agonist was used. • Check agonist dilutions for errors. • Make sure the agonist was present in the wells when the cells were injected. • Make sure the agonist dilution contains less than 5%DMSO final concentration
EC_{50} values are significantly right- or left-shifted	• Check the agonist dilution. • Check the setting of the reader. • Use the recommended media with BSA. Agonists may stick to plastic in absence of BSA. • Make sure the correct agonist was used. • Use fresh ligand dilutions. • Make sure that cells are not clumping together in the wells.
Some wells are showing much higher or much lower values	• Occasional spikes due to dust or contamination are normal. • Clean the instrument carefully with fresh washing solution. • Make sure tips are not clogged • Make sure all wells have the good volume of ligand • Make sure the ligand drops are in the bottom of the wells.
Unusual variability	• Check pipets, multichannel pipets and instrument-related liquid handling for possible malfunctions. • During loading, keep cells at or below room temperature. • Make sure that cells are not clumping together in the wells. • Do not centrifuge or vortex aequorin cells after coelenterazine loading.

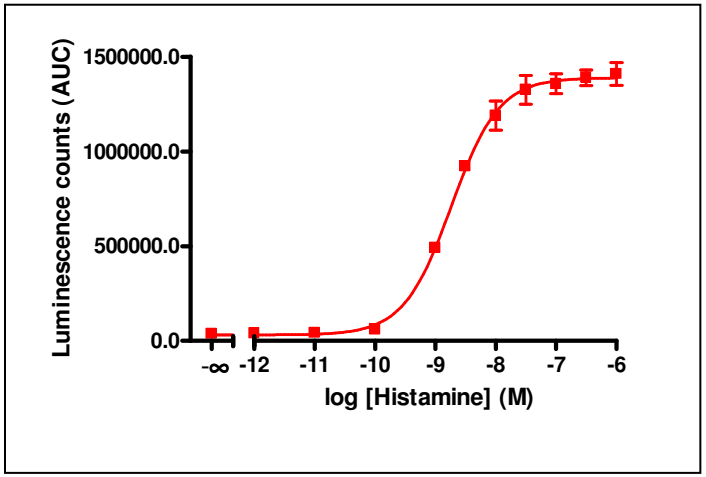
X. Appendix: Quick chart VICTOR

For VICTOR^{3}™, VICTOR^{3}™ V, VICTOR™ Light (with dispenser)

Typical results:

H1: Agonist Response on the VICTOR

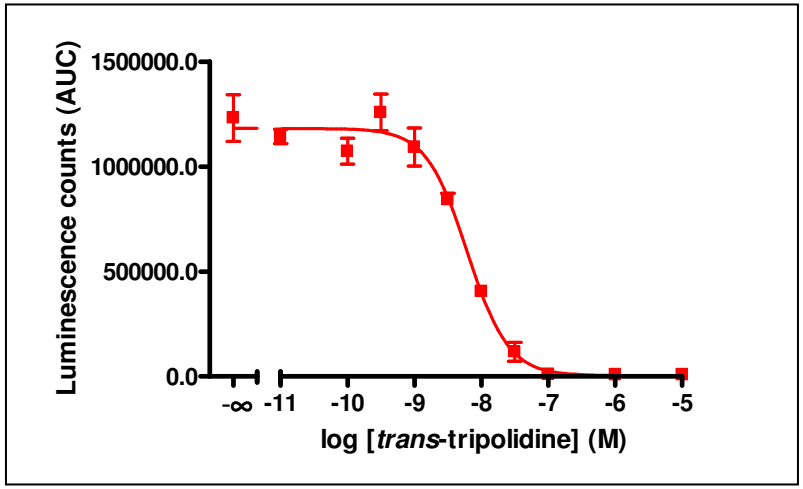
CHO-H_1 agonist assay, 96-well suspension (12,500 cells/well)



Agonist	pEC_{50} (M)	Digitonin (AUC)	Signal: Background	% Digitonin Response
Histamine	8.73	1396000	58.01	100.33

H1: Antagonist Response on the VICTOR

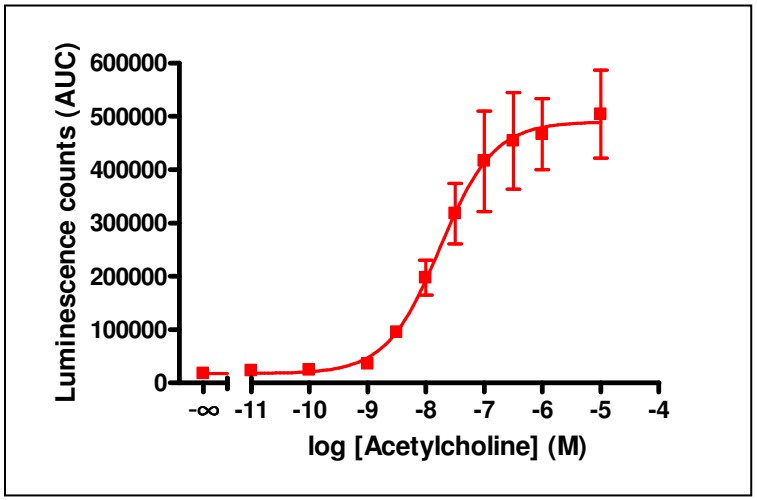
CHO-H_1 antagonist assay, 96-well suspension (12,500 cells/well).



Antagonist	pIC_{50} (M)
trans-tripolidine	8.08

M5: Agonist Response on the VICTOR

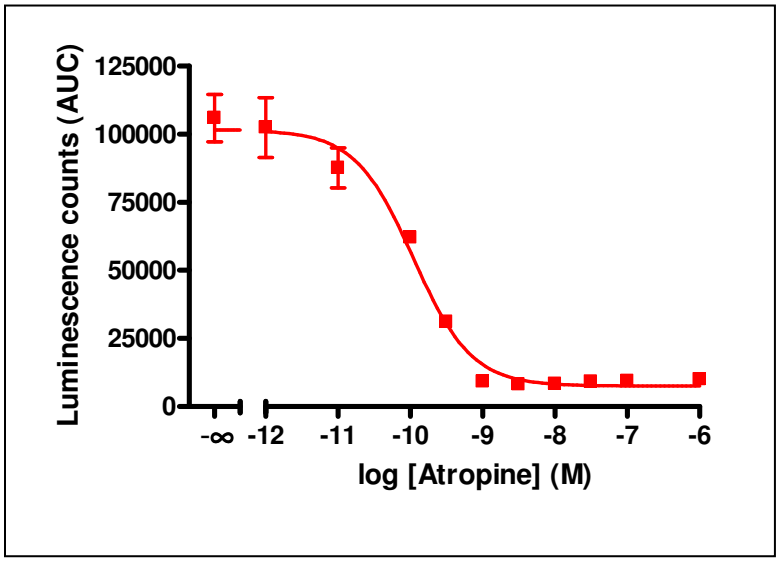
CHO-M_5 agonist assay, 96-well suspension (25,000 cells/well).



Agonist	pEC_{50} (M)	Digitonin (AUC)	Signal: Background	% Digitonin Response
Acetylcholine	7.76	805037	24.81	60.37

M5: Antagonist Response on the VICTOR

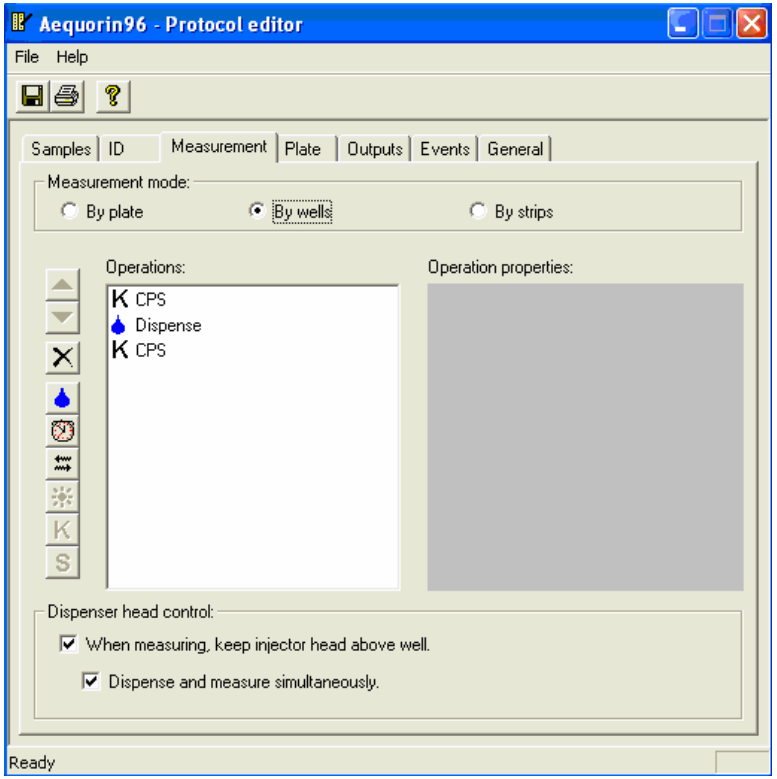
CHO-M_5 antagonist assay, 96-well suspension (25,000 cells/well).



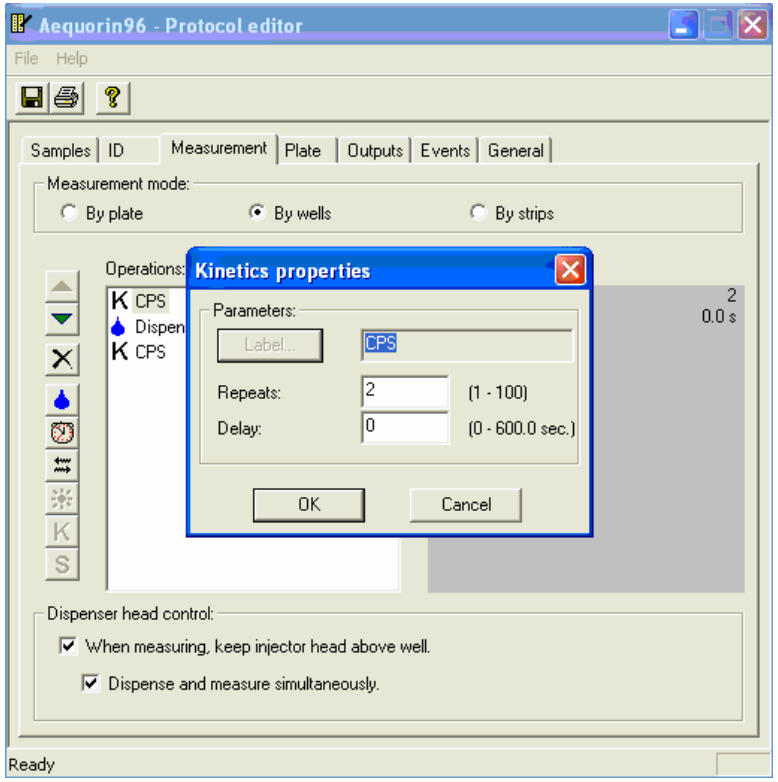
Antagonist	pIC_{50} (M)
Atropine	9.72

Instrument Settings:

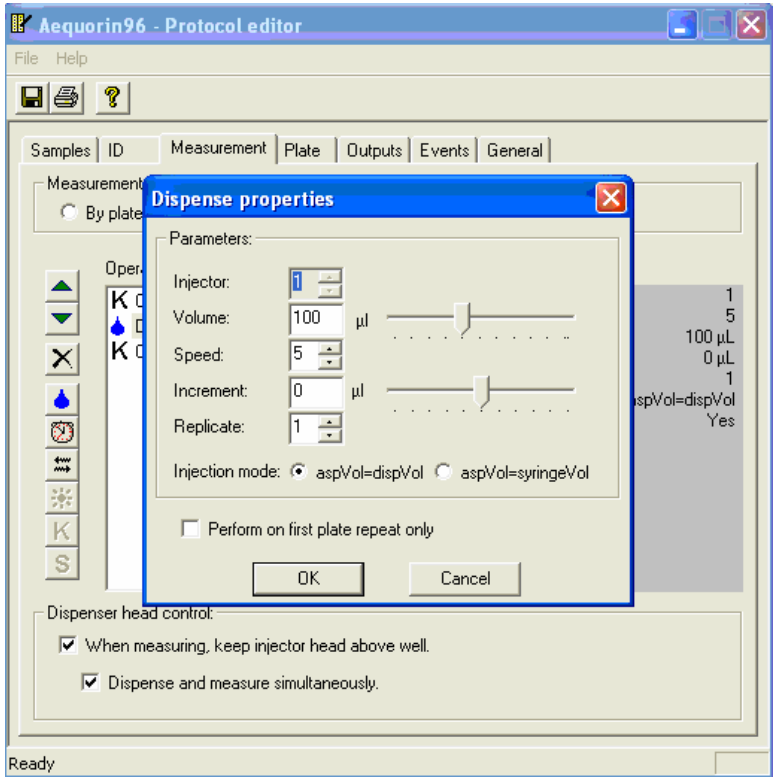
Protocol name	Aequorin96
Name of the plate type	OptiPlate 96
Number of wells in the plate	8 X 12
Height of the plate	14.6 mm
Offset of the wells	11.240 mm, 14.380 mm
Distance between wells	9.000 mm, 9.000 mm
Number of repeats	1
Delay between repeats	0 s
Measurement height	13.00 mm
Kinetic repeats	2
Kinetic delay	0.0 s
Name of the label	CPS
Label technology	Luminometry
Emission filter name	No filter
Emission filter slot	A7
Measurement time	1.0 s
Emission aperture	Normal
Injector	1
Speed	5
Volume	100 µL
Increment	0 µL
Replicate	1
Injection mode	aspVol=dispVol
Repeated operation	Yes
Kinetic repeats	20
Kinetic delay	0.0 s
Name of the label	CPS
Label technology	Luminometry
Emission filter name	No filter
Emission filter slot	A7
Measurement time	1.0 s
Emission aperture	Normal



Protocol set up



Baseline set up



Dispense set up

The second kinetic read (the response after dispense) is set up the same way as the baseline only with more repeats, typically 20 - 30.

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