human Adrenergic α_{2A} Receptor Aequorin Cell Line

Product No.: ES-030-A

Lot No.: 481-737-A

Material Provided

Cells:	2 x 1 mL frozen aliquot (ES-030-AV)
Format:	~2.5 x 10^6 cells /mL in freezing medium

Product Information

Cellular Background:	CHO-K1
Cell Line Development:	Our proprietary bicistronic expression plasmid containing the sequence coding for the human Adrenergic α_{2A} receptor was transfected in CHO-K1 cells stably expressing the mitochondrially targeted Aequorin and $G_{\alpha 16}$. Geneticin-resistant clones were obtained by limit dilution and compared for their response to a reference agonist using the AequoScreen® assay.
DNA Sequence:	Identical to coding sequence of GenBank NM_000681.2
Corresponding Protein Sequence:	Identical to GenBank NP_000672.2
Receptor expression level (B_{max}):	Estimated to be 8 pmol/mg protein, using [^{3}H]Rauwolscine.
K_d for the above radioligand:	1.2 nM
Shipping Conditions:	Shipped on dry ice. Please ensure dry ice is still present in the package upon receipt or contact Customer Support.
Storage Conditions:	Store in liquid nitrogen (vapor phase) immediately upon receipt.

Quality Control

The EC_{50} for a reference agonist was determined in an AequoScreen® assay performed on a MicroBeta® JET instrument. A mycoplasma test was performed using MycoAlert® Mycoplasma (Lonza) detection kit. We certify that these results meet our quality release criteria.

UK 14,304 (EC_{50}):	5.4 nM
Stability:	Cells were kept in continuous culture for at least 60 days and showed no decrease in functional response (EC_{50}, E_{max}).
Mycoplasma:	This cell line tested negative for mycoplasma.

Assay Procedures

We have shown for many of our GPCR cell lines that freshly thawed cells respond with the same pharmacology as cultured cells. All of our products validated in this way are available as frozen ready-to-use cells in our catalogue. Revvity also offers a custom service for the preparation of large quantities of frozen cryopreserved cells either from a catalogue cell line or a customer's own cell line. This demonstrates that cells can be prepared and frozen in advance of a screening campaign simplifying assay logistics.

Recommended Cell Culture Conditions (CHO-K1)

- The recommended media catalogue number and supplier reference information are listed in this Product Technical Data Sheet (last page). Media composition is specifically defined for each cell type and receptor expression selection. The use of incorrect media or component substitutions can lead to reduced cell viability, growth issues and/or altered receptor expression.
- Cells undergo major stress upon thawing, and need to adapt to their new environment which may initially affect cell adherence and growth rates. The initial recovery of the cells, and initial doubling time, will vary from laboratory to laboratory, reflecting differences in the origin of culture media and serum, and differences in methodology used within each laboratory.
- For the initial period of cell growth (i.e. until cells have reached Log-phase, typically 4-10 days), we strongly recommend removal of the antibiotics (G418, Zeocin™, Puromycin, Blasticidin, Hygromycin, Penicillin and Streptomycin) from the culture media. Immediately after thawing, cells may be more permeable to antibiotics, and a higher intracellular antibiotic concentration may result as a consequence. Antibiotics should be re-introduced when cells have recovered from the thawing stress.

Growth Medium:	Ham's F-12, 10% FBS, 0.4 mg/mL Geneticin (receptor expression selection), 0.25 mg/mL Zeocin (Aequorin and $G_{\alpha 16}$ expression selection).
Freezing Medium:	Ham's F-12, 10% FBS with 10% DMSO, without selection agents.

Thawing Cells: Using appropriate personal protective equipment, rapidly place the frozen aliquot in a 37°C water bath (do not submerge) and agitate until its content is thawed completely. Immediately remove from water bath, spray aliquot with 70% ethanol and wipe excess. Under aseptic conditions using a sterile pipette, transfer content to a sterile centrifuge tube containing 10 mL growth medium without antibiotics, pre-warmed at 37°C, and centrifuge (150 x g, 5 min). Discard supernatant using a sterile pipette. Resuspend cell pellet in 10 mL of pre-warmed growth medium without antibiotics by pipetting up and down to break up any clumps, and transfer to an appropriate culture flask (e.g. T-25, T-75 or T-175, see recommended seeding density below). Cells are cultured as a monolayer at 37°C in a humidified atmosphere with 5% CO_2.

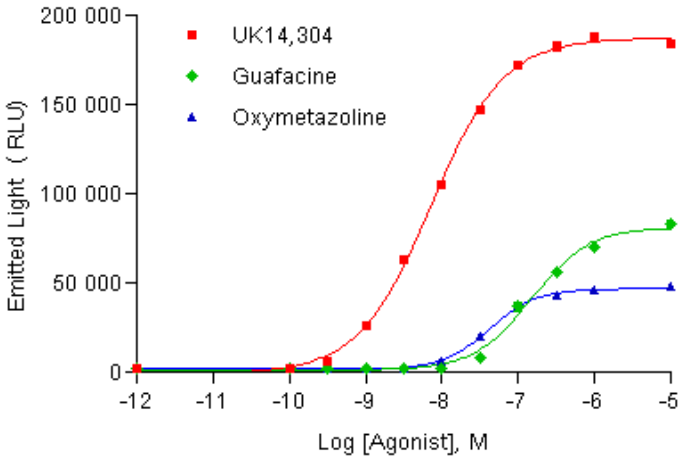
Recommended Seeding Density:	Thawing:	15000 – 33000 cells/cm^2
	Log-phase:	11000 – 15000 cells/cm^2

Troubleshooting: Initial doubling time can vary between 18 and 96 hours (Average = 25 hours). If cells are still not adhering after 48 hours or grow very slowly, we recommend maintaining the cells in culture and not replacing the media before 5-6 days (cells secrete factors that can help with adherence and growth). If confluence is still <50% after 5-6 days, it is recommended that you replace the media with fresh media (without antibiotics). Do not passage the cells until they reach 80-90% confluence (Log-phase). If cells have not recovered after 10-12 days, please contact our Technical Support.

Culture Protocol: Under aseptic conditions, cells are grown to 80% confluence (Log-phase) and trypsinized (0.05% trypsin/0.5 mM EDTA in calcium and magnesium-free PBS). See recommended seeding density for Log-phase above.

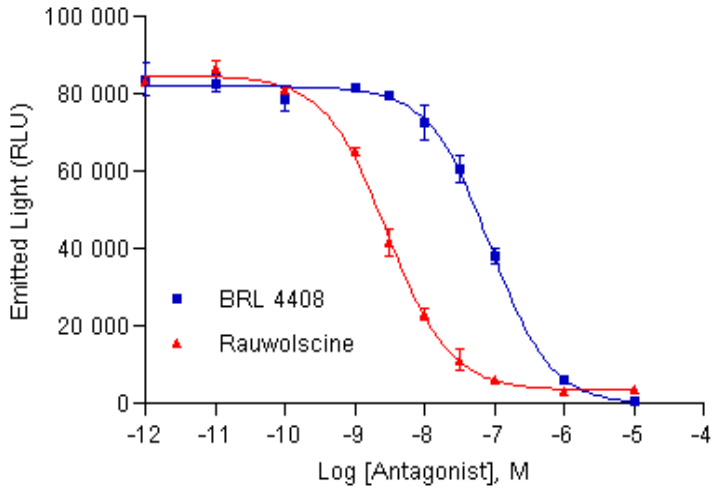
Banking Protocol: Cells are grown to 70-80% confluence (Log-phase). Under aseptic conditions, remove medium and rinse the flask with an appropriate volume of calcium and magnesium-free PBS (example 10 mL for T-175). Trypsinize (0.05% trypsin/0.5 mM EDTA in calcium and magnesium-free PBS) to detach cells (example 5 mL for T-175), let stand 5-10 min at 37°C. Add fresh, room temperature growth medium (without antibiotics) to stop trypsinization and dilute EDTA (example 10 mL for T-175). Transfer cells to a sterile centrifuge tube and centrifuge (150 x g, 5 min). Discard supernatant using a sterile pipette. Resuspend cell pellet in ice-cold freezing medium by pipetting up and down to break up any clumps. Count cells and rapidly aliquot at the selected cell density (e.g. 2.5 x 10^6 cells/mL) in sterile polypropylene cryovials. Use appropriate material to ensure slow cooling (about -1°C/min) until -70°C. Transfer vials into a liquid nitrogen tank (vapor phase) for storage.

Typical Product Data – AequoScreen® Assay



Agonist	EC50 (M)	% Digitonin response
UK14.304	7.3 X 10-9	80
Oxymetazoline	4.3 X 10-8	20
Guanfacine	1.5 X 10-7	35

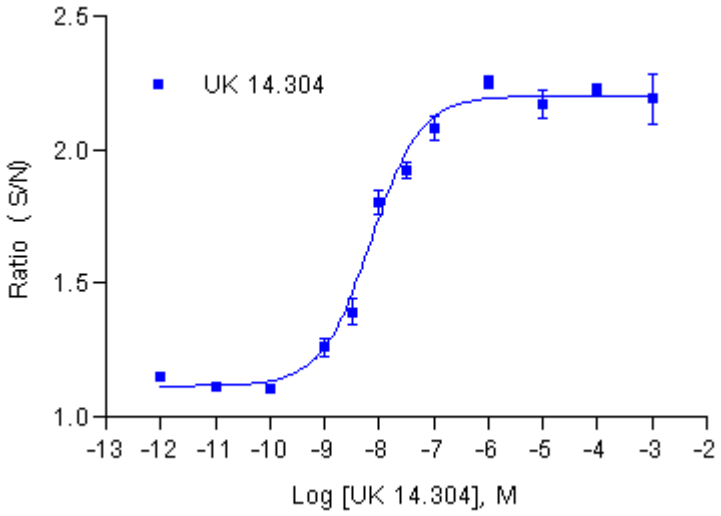
Figure 1: Agonist Response in AequoScreen® assay
An agonist dose-response experiment was performed in 96-well format using 25000 cells/well. Luminescence was measured on a MicroLumat Plus (Berthold). Data from a representative experiment are shown.



Antagonist	IC_{50} (M)
Rauwolscine	**3.0 x 10-9**
BRL 4408	**8.8 x 10-8**

Figure 2: Antagonist Response in AequoScreen® assay
An antagonist dose-response experiment was performed in 96-well format using 25000 cells/well with the reference agonist UK 14.304 injected at a final concentration equivalent to the EC_{80} (50 nM). Luminescence was measured on a MicroLumat Plus (Berthold). Data from a representative experiment are shown.

Typical Product Data – Calcium Assay (Fluorescence)



Agonist	EC_{50} (M)
UK 14.304	**7.1×10^{-9}**

Figure 3. Agonist Response in Fluo-4 Calcium assay
An agonist dose-response experiment was performed in 96-well format using 25 000 cells/well. Fluorescence was measured on a FDSS6000 (Hamamatsu Photonics). Data from a representative experiment are shown.

Typical Product Data –Radioligand Binding Assay (Filtration)

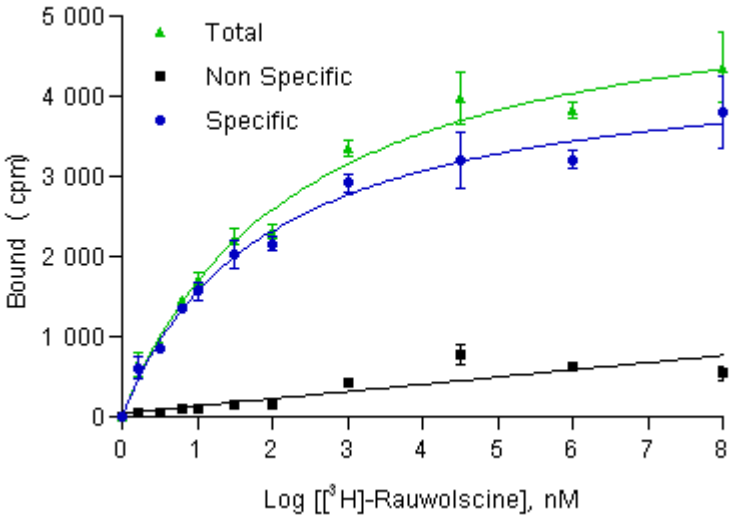


Figure 4: Saturation Binding Assay Curve (Filtration)
A saturation binding assay was performed in polyethylene MiniSorp (Nunc) tubes format using 6 µg membranes/tube. Counts per minute (CPM) were measured on the TopCount®. Data from a representative experiment are shown.

AequoScreen® Assay Procedure (MicroBeta® JET)

Assay Buffer:	DMEM / HAM's F12 with HEPES, without phenol red (Invitrogen # 11039-021) + 0.1 % protease-free BSA (from 10% solution sterilized by filtration at 0.22 µm). Store at 4°C.
Coelenterazine h:	To prepare a 500 µM Coelenterazine h stock solution, solubilize 250 µg of Coelenterazine h (Promega # S2011 or Invitrogen # C6780) in 1227 µL methanol. Store at –20°C in the dark.
Digitonin:	To prepare a 50 mM Digitonin stock solution, dissolve 1 g of Digitonin (Sigma # D5628) in 16.27 mL of DMSO. Aliquot and store at -20°C.

1. Cell Culture and Harvesting:	Grow cells (mid-log phase) in culture medium without antibiotics for 18 hours, Detach gently with PBS/0.5 mM EDTA, pH 7.4. Recover by centrifugation. Resuspend in Assay Buffer at a concentration of $3x10^5$ cells/mL.
2. Coelenterazine Loading:	Under sterile conditions, add "Coelenterazine h" at a final concentration of 5 µM to the cell suspension, mix well. Incubate at room temperature protected from light and with constant gentle agitation for at least 4 hours (incubation can be extended overnight).
3. Cell Dilution:	Dilute cells 3x in assay buffer and incubate as described above for 60 min.
4. Ligands and plates preparation:	Prepare serial dilutions of ligands in assay buffer (2x concentration for agonists, 2x concentration for antagonists). Dispense 50 µL of diluted ligand in a 96-well Optiplate™. *Note: Assay can be miniaturized to 384-well and 1536-well formats.*
5. Agonist Mode Reading:	Using the reader's automatic injection system, inject 50 µL of cells (i.e. 5 000 cells) per well and immediately record relative light emission for 20-40 seconds. Digitonin at a final concentration of 100 µM in assay buffer is used in control wells to measure the receptor independent cellular calcium response.
6. Antagonist Mode Reading:	After 15 minutes of incubation of the cells with the ligand, using the reader's automatic injection system, inject 50 µL of the reference agonist at a final concentration equivalent to the EC_{80} and immediately record relative light emission for 20-40 seconds.
7. Data Analysis:	Sigmoidal dose-response curves are generated using average Luminescent Counts Per Second (LCPS) recorded for 20-40 sec immediately after cells are mixed with the agonist in agonist mode or the EC_{80} of a reference agonist in antagonist mode.

Important Notes:

- Temperature should remain below 25°C during the coelenterazine loading of the cells, and until using the cells for the readings. Excessive heating by the cell stirrer for example will result in signal loss.
- Depending on (1) sensitivity of the reader used, (2) plate format used, and (3) assay characteristics wanted, it is possible to load cells at (a) different concentrations of cells and coelenterazine, (b) with different subsequent dilution factors, and (c) using different cell numbers per well. This is part of the validation work when importing an assay to a new reader.
- For tips and examples on running AequoScreen® assays on different readers, please refer to the AequoScreen® Starter Kit Manual available at www.revvity.com.

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Calcium Assay Procedure (Fluorescence)

Dye solution: 5 µM Fluo-4 AM (Molecular Probes, P-6867), 1 mg/mL Pluronic acid in Assay Buffer

Assay Buffer: 2.5 mM Probenicid, 0.1% BSA, 0.05% Gelatin, 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 5.6 mM Glucose, pH 7.4

Controls: Maximal Signal: 0.4% Triton X-100 (0.2% final) in Assay Buffer
Minimum signal: 0.4% Triton X-100 (0.2% final), 20 mM EGTA (10 mM final) in Assay Buffer

Reader: FDSS 6000 (Hamamatsu Photonics), Excitation 480 nm / Emission 520 to 560 nm, 96-well

Day 1		
1. Cell Culture and Harvesting:	Grow cells (mid-log phase) in culture medium without antibiotics for 18 hours, Detach gently with PBS/0.5 mM EDTA, pH 7.4, Recover by centrifugation, Resuspend in medium without antibiotics at $2.5x10^5$ cells/mL.	
2. Cell Seeding	Distribute 100 µL (i.e. 25,000 cells) in each well of a 96 well black, clear bottom TC sterile plate, incubate overnight in a cell culture incubator (37°C, 5% CO_2).	
Day 2		
3. Cell Loading	Remove the media and add 100 µL/well of Dye solution.	
4. Incubation	Incubate the assay plate for 1 hour at 37°C in a cell culture incubator.	
5. Ligands and compound plates preparation:	Prepare serial dilutions of 2x concentrated ligands in Assay Buffer, Dispense 100 µL/well of diluted ligand in a 96-well plate. *Note: Assay can be miniaturized to 384-well format.*	
6. Dye Washing	Drain the media and wash the wells twice with 100 µL/well Assay Buffer,	
7. Buffer/Antagonist addition	Agonist assay: Add Assay Buffer to make a total of 50 µL	Antagonist Assay: Add 2x antagonist dilution in Assay Buffer to make a total of 50 µL
8. Equilibration	Incubate the plate for 20 min at room temperature in the dark.	
9. Plate Reading:	Using the reader's injection system, inject 50 µL per well of 2x agonist solutions in Assay Buffer, and immediately record relative light emission for 90 seconds.	Using the reader's injection system, inject 50 µL per well of 2x concentrated reference agonist in Assay Buffer (final EC_{80} concentration), and immediately record relative light emission for 90 seconds.
10. Data Analysis:	The fluorescent signal is expressed as the ratio relative to the first measurement (i.e. before dispensing), and the maximal value of this ratio during the measurement interval is used to draw sigmoidal dose-response curves.	

Important Notes:

- Probenicid is prepared as a 250 mM solution in a 50:50 mixture of 1N NaOH : Assay Buffer.

Membrane Radioligand Binding Assay Procedure (Filtration)

Note: The following are recommended assay conditions and may differ from the conditions used to generate the typical data shown in the above section.

Assay Buffer: 50 mM TRIS-HCl pH 7.4, 1 mM EDTA, 12.5 mM $MgCl_2$

Wash Buffer: 50 mM TRIS-HCl pH 7.4, 12.5 mM $MgCl_2$

Radioligand: [^{3}H]Rauwolscine (Revvity # NET722)

Filters: Unifilter 96 GF/C (Revvity # 6055690)

Membrane Binding Protocol:

Binding assays were performed in 550 µL total volume according to the following conditions. All dilutions are performed in assay buffer:

1. Membrane dilution:	1 µg of membranes per well, diluted in order to dispense 500 µL/well. Keep on ice.
2. Assembly on ice (in 96 Deep well plate) Saturation Binding:	• 25 µL of assay buffer or of unlabeled ligand (UK 14,304, 1 to 2 µM final to have at least a 200-fold excess compared to [^{3}H]Rauwolscine) for determination of non specific binding • 25 µL of radioligand at increasing concentrations (see figure 4) • 500 µL of diluted membranes
Competition Binding:	• 25 µL competitor ligand at increasing concentrations • 25 µL of radioligand (2.5 nM final) • 500 µL of diluted membranes
3. Incubation:	60 min at 27 °C.
4. Filters preparation:	GF/C filters were presoaked in 0.5 % PEI at room temperature for at least 30 min.
5. Filtration:	Aspirate and wash 9 x 500 µL with ice cold wash buffer using a FilterMate Harvester.
6. Counting:	Add 30 µL/well of MicroScint™-O (Revvity # 6013611), cover filter with a TopSeal-A PLUS (Revvity # 6050185) and read on a TopCount®.

References

1. Dupriez VJ, Maes K, Le Poul E, Burgeon E, Detheux M. (2002) Aequorin-based functional assays for G-protein-coupled receptors, ion channels, and tyrosine kinase receptors. Receptors Channels 8:319-30

2. Rizzuto R. Simpson AWM, Brini M, Pozzan T. (1992) Rapid changes of mitochondrial Ca^{2+} revealed by specifically targeted recombinant aequorin. Nature 358:325-327.

3. Stables J., Green A., Marshall F., Fraser N., Knight E., Sautern M., Milligan G., Lee M., Rees S. (1997) A bioluminescent assay for agonist activity at potentially any G-protein-coupled receptor. Anal. Biochem. 252:115-126.

4. Milligan G, Marshall F, and Rees S. (1996) $G_{\alpha 16}$ as a universal G protein adapter: implications for agonist screening strategies. TIPS 17:235-237.

5. Offermanns S, Simon M. (1995) $G_{\alpha 15}$ and $G_{\alpha 16}$ couple a wide variety of receptors to phospholipase C. J. Biol. Chem. 270:15175-15180.

6. FraserCM, Arakawa S, McCombie WR, Venter JC. (1989) Cloning, sequence analysis, and permanent expression of a human alpha 2-adrenergic receptor in Chinese hamster ovary cells. Evidence for independent pathways of receptor coupling to adenylate cyclase attenuation and activation. J. Biol. Chem., 264, 11754-11761

7. Kobilka BK, Matsui H, Kobilka TS, Yang-Feng TL, Francke U, Caron MG, Lefkowitz RJ, Regan JW. (1987) Cloning, sequencing, and expression of the gene coding for the human platelet alpha 2-adrenergic receptor. Science, 238, 650-656

Materials and Instrumentation

The following tables provide the references of compounds and reagents used for the characterization of the human Adrenergic α_{2A} receptor Aequorin cell line, as well as some advice on how to use these compounds:

Table 1. References of compounds used for functional characterization and binding assays

Name	Provider	Cat no	Working Stock Solution
UK 14,304	Tocris	0425	100 mM in DMSO
Oxymetazoline	Sigma	O2378	10 mM in H_2O
Guanfacine	Tocris	1030	100 mM in H_2O
Rauwolscine	Tocris	0891	1 mM in H_2O
BRL 44408 maleate	Tocris	1133	10 mM in DMSO
[^{3}H]Rauwolscine	Revvity	NET722	N/A

Table 2. References of cell culture media and assay buffers.

Note: The table below lists generic media and additives typically used for Revvity cell lines. For product specific media and additives, please refer to the "Recommended Cell Culture Conditions" section.

Name	Provider	Cat no
HAM's F-12	Hyclone	SH30026.02
DMEM	Hyclone	SH30022.02
Advanced DMEM/F12 (serotonin receptors)	Invitrogen	12634-010
EMEM	BioWitthaker	06-174G
EX-CELL DHFR$^-$ media (DHFR deficient cell lines)	Sigma	C8862
FBS	Wisent	80150
FBS dialyzed	Wisent	80950
G418 (geneticin)	Wisent	400-130-IG
Zeocin	Invitrogen	R25005
Blasticidin	invitrogen	R210-01
Puromycin	Wisent	400-160-EM
Standard HBSS (with $CaCl_2$ and $MgCl_2$)	GIBCO	14025
HEPES	MP Biomedicals, LLC	101926
BSA, Protease-free	Sigma	A-3059
PEI	Sigma	P3143
Trypsin-EDTA	Hyclone	SH30236.02
Sodium Pyruvate	GIBCO	11360
L-Glutamine	GIBCO	25030
NEAA (non-essential amino acids)	GIBCO	11140

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