human Adrenergic α_{1A} Receptor Cell Line

Product No.: ES-036-C

Lot No.: 1815279

Material Provided

Cells:	2 x 1 mL frozen aliquot (ES-036-CV)
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_{1A} receptor was transfected in CHO-K1 cells. Geneticin-resistant clones were obtained by limit dilution and compared for receptor expression levels by radioligand binding assay. The clone with the highest receptor expression level was selected for characterization in binding and functional assays.
DNA Sequence:	Identical to coding sequence of GenBank NM_000680.2.
Corresponding Protein Sequence:	Identical to GenBank P35348.2.
Receptor expression level (B_{max}):	Estimated to be 53 pmol/mg protein, using [^{125}I]HEAT
K_d for the above radioligand:	2.9 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 IP Accumulation assay. A mycoplasma test was performed using MycoAlert® (Lonza) mycoplasma detection kit. We certify that these results meet our quality release criteria.

A61603 (EC_{50}):	N/D
Stability:	Cells were kept in continuous culture for at least 60 days and showed no decrease of receptor expression level in a saturation binding assay (stable B_{max} and K_d) and no decrease in functional response (EC_{50}, E_{max} in cAMP assay).
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. 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 G418 (receptor 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.

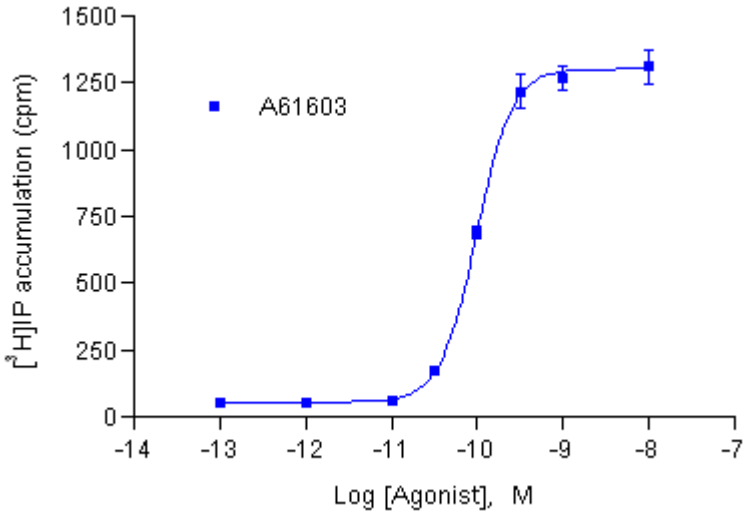
Recommended Seeding Density:	Thawing:	15,000 – 33,000 cells/cm^2
	Log-phase:	11,000 – 15,000 cells/cm^2

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 – Inositol Phosphate Assay



Agonist	EC_{50} (M)
A 61603	9.7×10^{-11}

Figure 1. Agonist Response in IP Accumulation assay
An agonist dose-response experiment was performed in 24-well format seeding 60,000 cells/well 48 hours prior to the experiment. After growing overnight, cells were incubated with 3 µCi/ml [^{3}H] myo-inositol for 24 hours. Medium was then removed and cells were incubated for 30 minutes with different concentrations of agonist. After stopping the reaction, cells were detached, centrifugated and placed in mini anion exchange columns previously prepared (see protocol). The IPs mixture were eluted and collected in scintillation vials. Radioactivity was determined using a Tricarb counter. Data from a representative experiment are shown.

Typical Product Data –Radioligand Binding Assay (Filtration)

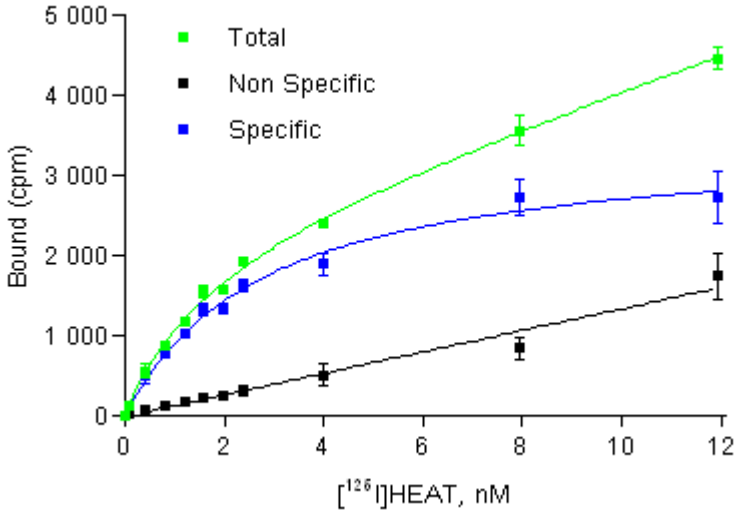
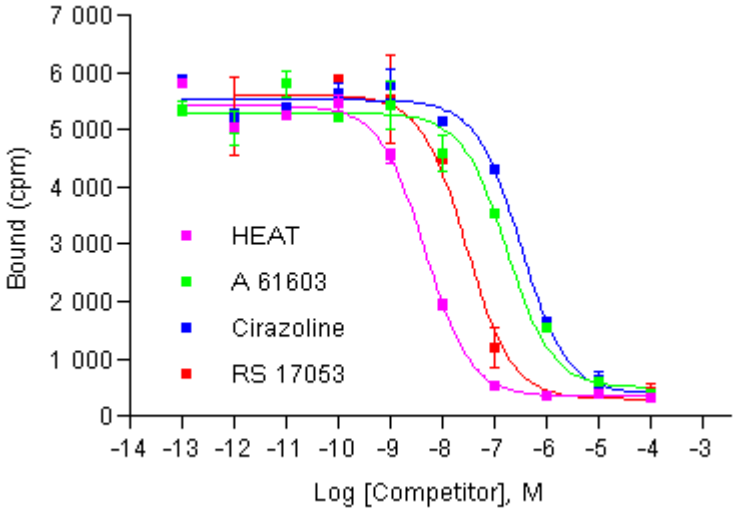


Figure 2: Saturation Binding Assay Curve (Filtration)
A saturation binding assay was performed in 96-well format using 0.1 µg membranes/well. Counts per minute (cpm) were measured on a TopCount® instrument. Data from a representative experiment are shown.



Agonist / Antagonist	IC_{50} (M)
HEAT	4.7×10^{-9}
A 61603	1.7×10^{-7}
Cirazoline	3.2×10^{-7}
RS 17053	2.9×10^{-8}

Figure 3: Competition Binding Assay Curve (Filtration)
A competition binding assay was performed in 96-well format using 0.1 µg membranes/well. Displacement of 0.35 nM [125I]HEAT was used. Counts per minute (cpm) were measured on a TopCount® instrument. Data from a representative experiment are shown.

revvity

IP Assay Procedure (SPA)

Loading Medium:	DMEM, w/o L-Glut., w/o i-Inositol (ICN # 1642954), 20 µCi/mL Inositol, myo-[2-^{3}H(N)]- (Revvity # NET114A), 2 mM L-glutamine, 0.3 % protease-free BSA.
Stimulation Medium:	Loading Medium + 10 mM LiCl.
Lysis Buffer:	0.1 M formic acid in water.
SPA Beads suspension:	Dilute RNA Binding Beads (YSI) (Revvity # RPNQ0013) at 5.55mg/mL in dH_2O

Day 1	
1. Cell Culture and Harvesting:	Grow cells (mid-log phase) in culture medium without antibiotics for 18 hours. Recover cells by trypsinization and centrifugation, resuspend in culture medium with 10% FBS, without antibiotics at 2.5x10^5 cells/mL.
2. Cell Seeding	Dispense 100 µL (i.e. 25,000 cells) in each well of a 96 well TC sterile plate, incubate overnight (37°C, 5% CO_2).
Day 2	
3. Cell Loading	Remove the media, wash twice (2 x 100 µL) with inositol-free DMEM and add 100 µL/well of Loading Medium.
4. Incubation	Incubate the assay plate overnight (37°C, 5% CO_2).
Day 3	
5. Ligands and compound plates preparation:	Add 10 mM LiCl to an aliquot of Loading Medium (pre-heated to 37°C) to prepare Stimulation Medium. Prepare serial dilutions of 2x concentrated ligands in Stimulation Medium.
6. Cells Stimulation:	Remove the Loading Medium, wash twice (2 x 100 µL) with pre-heated Loading Medium and add 100 µL/well of Ligands dilutions prepared in Stimulation Medium. Incubate for 30 min at 37°C.
7. Cell Lysis:	Remove the medium, add 100 µL/well of Lysis Buffer. Incubate for 20 min at RT.
8. SPA assay assembly:	In a 96-well white Optiplate, dispense 90 µL of the Beads suspension per well (i.e. 0.5 mg Beads/well). *Note: keep stock of beads in suspension.* Gently shake the cell plate by inclining it 5 to 10 times, Aspirate 10 µL of cellular lysate (avoid touching the cells or pipeting up and down), and dispense on top of the 90 µL of Beads prepared above. Add a TopSeal, and incubate for 1 h at RT with plate shaking.
9. Plate Reading:	Incubate at least 1 additional hour without shaking (can be incubated overnight) before reading the plate. read on a TopCount® instrument.
10. Data Analysis:	The cpm measured are used to draw a sigmoidal dose-response curve.

Inositol Phosphate Accumulation Assay

Cells were seeded 2 days before the experiment ($6x10^4$/well in a 24-well plate). After growing overnight in the recommended culture medium, the medium was changed to DMEM (Biowhittaker, # 12604F) containing 3 µCi/ml [^{3}H] myo-inositol. The following day, medium containing [^{3}H] myo-inositol was removed and cells were washed twice with CSS buffer (25 mM Tris, pH 7.4; 120 mM NaCl; 5.4 mM KCl; 1.8 mM $CaCl_2$; 0.8 mM $MgCl_2$; 16.4 mM D-glucose) and incubated for 30 min at 37°C with various concentrations of agonist in the CSS buffer containing 10 mM of LiCl. Reactions were stopped by addition of 1N $HClO_4$ to each well and incubated for 30 min at 4°C. KOH phosphate buffer was then added to each well and incubated at 4°C for 1 hour to form $KClO_4$ precipitate. A sample buffer containing 30 mM DiNatetraborate and 3 mM EDTA was then added on each well and the plate was centrifuged at 1500 rpm at 4°C for 5 min.

At the same time, mini anion exchange columns were prepared as follows. Dowex resin AG1X8 formate form (Biorad, # 140-1454) was weighted and mixed with distilled water (25% w/v). 1.6 ml of this resin was added to each Poly Prep column (Biorad, # 731-1550). The columns were then washed one time with 5 ml of distilled water. The supernatant of each well was applied to column containing anion exchange resin and the columns were rinsed with 5 ml of distilled water. The GPI was eluted with 10 ml of 5 mM Di Natetraborate / 60 mM Ammonium formate. The IPs mixture was then eluted with 0.1M formic acid/1M ammonium formate and collected into scintillation vials. Radioactivity was determined by adding 3.5 ml of scintillation coktail and counting on a Tri-Carb. EC_{50} were determined by non-linear regression using a single site model.

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:	25 mM Hepes pH 7.4, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5% BSA
Wash Buffer:	25 mM Hepes pH 7.4, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 500 mM NaCl (ice cold)
Radioligand:	[^{125}I]-HEAT (Revvity # NEX182)
Filters:	Unifilter 96 GF/C (Revvity # 6055690)

Membrane Binding Protocol:

Binding assays were performed in 200 µL total volume according to the following conditions. All dilutions are performed in assay buffer:

1. Membrane dilution:	0.2 µg of membranes per well, diluted in order to dispense 150µL/well. Keep on ice.
2. Assembly on ice (in 96 Deep well plate) Saturation Binding:	• 25 µL of assay buffer or of unlabeled ligand (HEAT, 2.4 µM final) for determination of non specific binding • 25 µL of radioligand at increasing concentrations (see figure 2) • 150 µL of diluted membranes
Competition Binding:	• 25 µL competitor ligand at increasing concentrations (see figure 3) • 25 µL of radioligand (0.2 nM final) • 150 µL of diluted membranes
3. Incubation:	30 min at 37°C.
4. Filters preparation:	GF/C filters were presoaked in 0.5% Brij 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. Ford AP, Daniels DV, Chang DJ, Gever JR, Jasper JR, Lesnick JD, Clarke DE. (1997) Pharmacological pleiotropism of the human recombinant alpha1A-adrenoceptor: implications for alpha1-adrenoceptor classification. Br J Pharmacol. 121:1127-1135.

2. Knepper SM, Buckner SA, Brune ME, DeBernardis JF, Meyer MD, Hancock AA. (1995) A-61603, a potent alpha 1-adrenergic receptor agonist, selective for the alpha 1A receptor subtype. J Pharmacol Exp Ther. 274:97-103.

3. Michel AD, Loury DN, Whiting RL. (1989) Identification of a single alpha 1-adrenoceptor corresponding to the alpha 1A-subtype in rat submaxillary gland. Br J Pharmacol. 98:883-889.

4. Shibata K, Hirasawa A, Moriyama N, Kawabe K, Ogawa S, Tsujimoto G.(1996) Alpha 1a-adrenoceptor polymorphism: pharmacological characterization and association with benign prostatic hypertrophy. Br J Pharmacol. 118:1403-1408.

Materials and Instrumentation

The following tables provide the references of compounds and reagents used or recommended for the characterization of the human Adrenergic α_{1A} receptor ValiScreen® 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
A 61603	Tocris	1052	10 mM in dH_2O
HEAT	Tocris	0535	10 mM in dH_2O
Cirazoline	Tocris	0888	10 mM in dH_2O
RS 17053	Tocris	0985	10 mM in DMSO
[^{125}I]HEAT	Revvity	NEX182	N/A

Table 2. References of cell culture media and assay buffers

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
Forskolin	Sigma	F6886

Revvity
940 Winter Street
Waltham, MA 02451 USA

(800) 762-4000
www.revvity.com

For a complete listing of our global offices, visit www.revvity.com