human Ghrelin Receptor Cell Line

Product No.: ES-410-C

Lot No.: M1W-C1

Material Provided

Cells:	2 x 1 mL frozen aliquot (ES-410-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 Ghrelin receptor was transfected in CHO-K1 cells. Geneticin-resistant clones were obtained by limit dilution and compared for receptor expression levels using a 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_198407.1
Corresponding Protein Sequence:	Identical to GenBank NP_940799.1.
Receptor expression level (B_{max}):	Estimated to be 22 pmol/mg protein, using [His-^{125}I] Ghrelin
K_d for the above radioligand:	0.2 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 a Calcium flux assay. A mycoplasma test was performed using MycoAlert® (Lonza) mycoplasma detection kit. We certify that these results meet our quality release criteria.

Ghrelin (EC_{50}):	1.5 nM
Stability:	Cells were kept in continuous culture for at 90 days and showed some decrease of receptor expression level in a saturation binding assay: The B_{max} value after 36, 64 and 90 days of continuous culture was 36, 18 and 10 pmol/mg protein respectively, while the K_d was unchanged. This decrease is most probably attributable to the constitutive activity of the receptor. We therefore do not recommend keeping the cell line in continuous culture more than one month.
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:	Advanced DMEM/F12, 1% FBS dialyzed, 4 mM L-Glutamine, 0.4 mg/ml Geneticin (receptor expression selection).
Freezing Medium:	Advanced DMEM/F12, 1% FBS dialyzed, 4 mM L-Glutamine, 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.

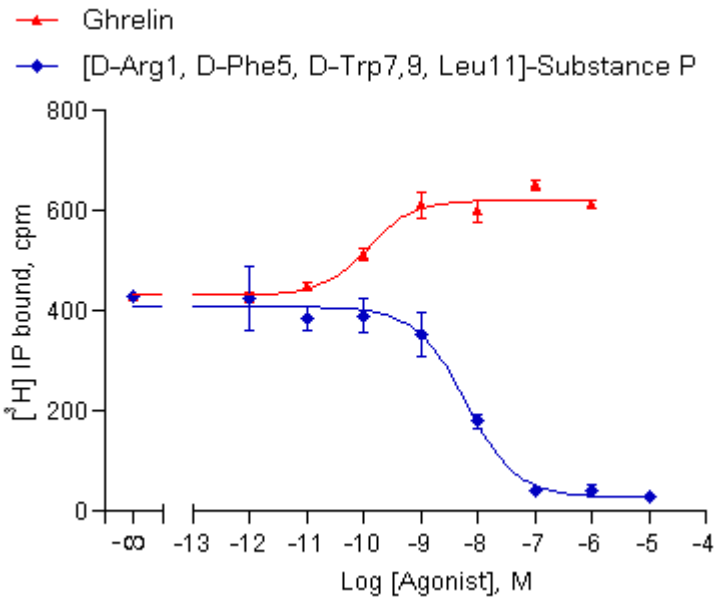
Recommended Seeding Density:	Thawing:	15,000 – 33,000 cells/cm^2
	Log-phase:	11,000 – 15,000 cells/cm^2

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.

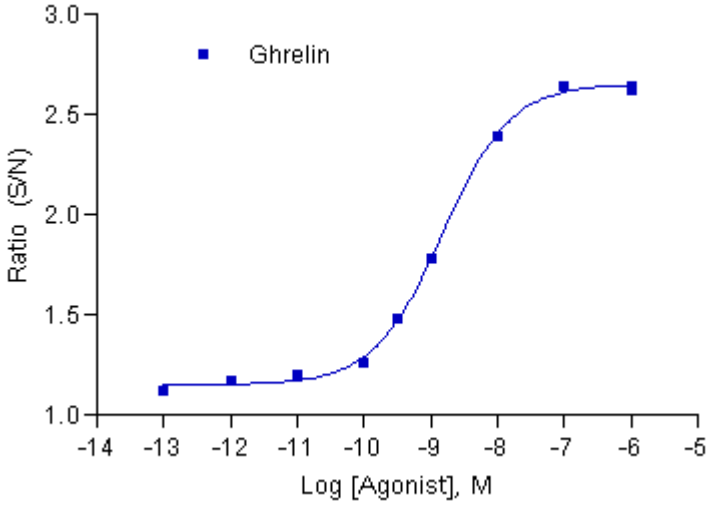
Typical Product Data – Inositol Phosphate Assay



Agonist	EC_{50} (M)
Ghrelin	**1.2×10^{-10}**
[D-Arg1, D-Phe5, D-Trp7,9, Leu11]-Substance P (Inverse Agonist)	**6.2×10^{-9}**

Figure 1. Agonist Response in IP -SPA assay
An agonist dose-response experiment was performed in 96-well format using 25,000 cells/well. After loading with [^{3}H] myo-inositol, cells were stimulated with the indicated agonist for 30 min, and then lysed. An aliquot of the cell lysate was incubated with SPA beads, and preferential binding of the formed inositol phosphates to the beads was used to detect IP formation in response to the agonist stimulation. Counts per minute (cpm) were measured on a TopCount® Instrument. Data from a representative experiment are shown.

Typical Product Data – Calcium Assay (Fluorescence)



Agonist	EC_{50} (M)
Ghrelin, human	**1.5×10^{-9}**

Figure 2. Agonist Response in Calcium Fluo-4 assay
An agonist dose-response experiment was performed in 96-well format using 25,000 cells/well. Fluorescence was measured on a FDSS 6000 (Hamamatsu Photonics). Data from a representative experiment are shown.

Typical Product Data –Radioligand Binding Assay (Filtration)

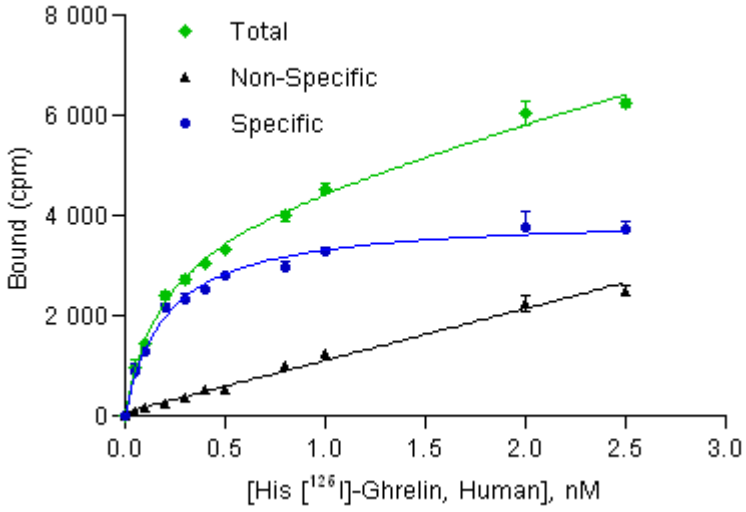
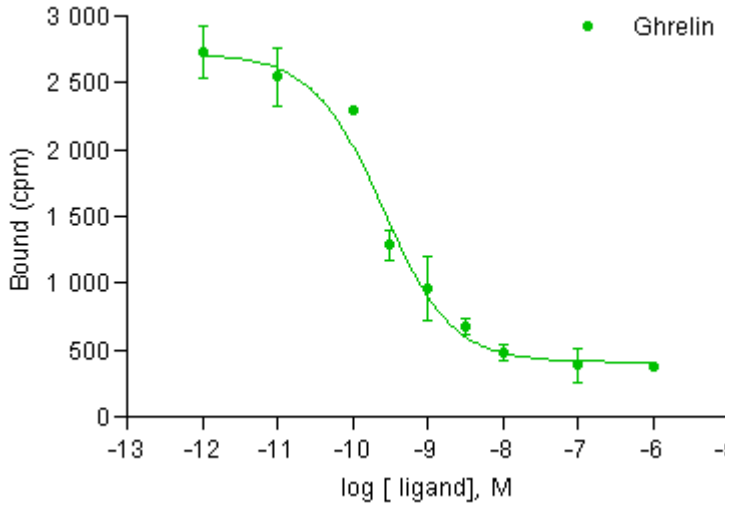


Figure 3: Saturation Binding Assay Curve (Filtration)
A saturation binding assay was performed in 96-well format using 0.5 µg membranes/well. Counts per minute (cpm) were measured on a TopCount® instrument. Data from a representative experiment are shown.



Agonist / Antagonist	IC_{50} (M)
human Ghrelin	2.5×10^{-10}

Figure 4: Competition Binding Assay Curve (Filtration)
A competition binding assay was performed in 96-well format using 0.5 µg membranes/well. Displacement of 0.2 nM [^{125}I]-Ghrelin was used. Counts per minute (cpm) were measured on a TopCount® instrument. Data from a representative experiment are shown.

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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, resuspended in usual 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.

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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: 25 mM Hepes pH 7.4, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5% BSA

Wash Buffer: 50 mM Tris-HCl pH 7.4

Radioligand: [^{125}I]-Ghrelin (Revvity # NEX388)

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:	1 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 (Ghrelin (human), 5 µM final) for determination of non specific binding • 25 µL of radioligand at increasing concentrations (see figure 3) • 150 µL of diluted membranes
  Competition Binding:	• 25 µL competitor ligand at increasing concentrations (see figure 4) • 25 µL of radioligand (0.033 nM final) • 150 µL of diluted membranes
3. Incubation:	30 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. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. (1999) Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature. 402:656-660.

2. Howard AD, Feighner SD, Cully DF, Arena JP, Liberator PA, Rosenblum CI, Hamelin M, Hreniuk DL, Palyha OC, Anderson J, Paress PS, Diaz C, Chou M, Liu KK, McKee KK, Pong SS, Chaung LY, Elbrecht A, Dashkevicz M, Heavens R, Rigby M, Sirinathsinghji DJ, Dean DC, Melillo DG, Patchett AA, Nargund R, Griffin PR, DeMartino JA, Gupta SK, Schaeffer JM, Smith RG, Van der Ploeg LH. (1996) A receptor in pituitary and hypothalamus that functions in growth hormone release. Science. 273:974-977.

3. Holst B, Cygankiewicz A, Jensen TH, Ankersen M, Schwartz TW. (2003) High constitutive signaling of the ghrelin receptor--identification of a potent inverse agonist. Mol Endocrinol. 17:2201-2210.

Materials and Instrumentation

The following tables provide the references of compounds and reagents used or recommended for the characterization of the human Ghrelin 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
Ghrelin (human)	Bachem	H-4864	100 µM in PBS / 0.1% protease-free BSA
[D-Arg1, D-Phe5, D-Trp7,9, Leu11]-Substance P	Phoenix Pharmaceuticals	061-19	1 mM in PBS / 0.1% protease-free BSA
[^{125}I]-Ghrelin	Revvity	NEX388	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