human Neuropeptide S Receptor, isoform A (NPSR1, GPRA) Cell Line

Product No.: ES-770-C

Lot No.: 546-454-A

Material Provided

Cells:	2 x 1 mL frozen aliquot (ES-770-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 Neuropeptide S Receptor, isoform A (NPSR1, GPRA) 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. Clones with various receptor expression levels were compared in a calcium flux assay, and the clone with the best response was selected.
DNA Sequence:	Identical to coding sequence of GenBank EA419043.1.
Corresponding Protein Sequence:	Identical to GenBank ABZ63404.1.
Receptor expression level (B_{max}):	Estimated to be 0.74 ± 0.34 pmol/mg protein, using [^{125}I]- Neuropeptide S
K_d for the above radioligand:	0.39 ± 0.11 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 Calcium flux assay performed on a CellLux® instrument. A mycoplasma test was performed using MycoAlert® (Lonza) mycoplasma detection kit. We certify that these results meet our quality release criteria.

Neuropeptide S (human) (EC_{50}):	6.7 nM
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 IP-ONE 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.

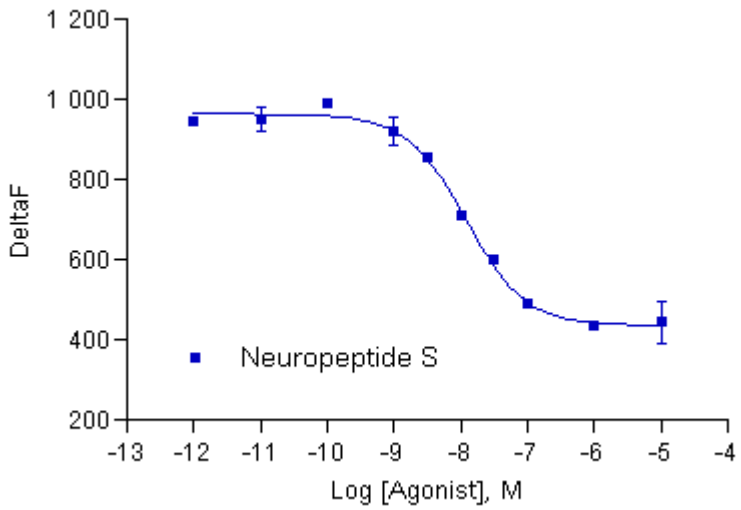
Recommended Seeding Density:	Thawing:	15,000 – 33,000 cells/cm^2
	Log-phase:	11,000 – 15,000 cells/cm^2

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.

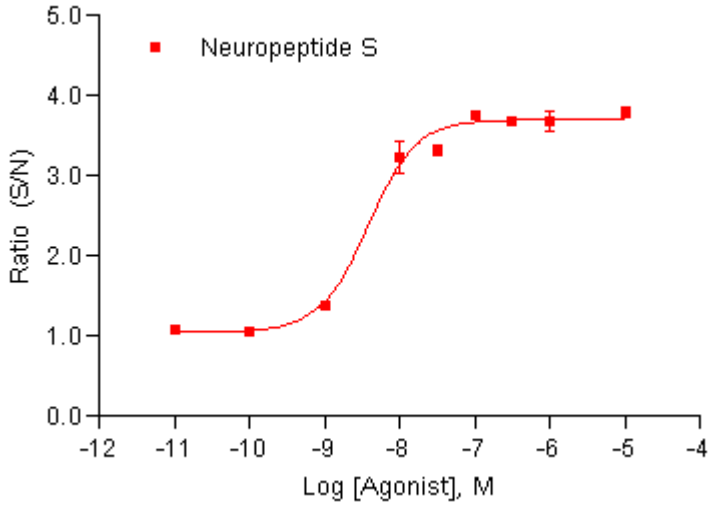
Typical Product Data – Inositol Phosphate Assay



Agonist	EC_{50} (M)
Neuropeptide S	1.2×10^{-8}

Figure 1. Agonist Response in IP-ONE assay
An agonist dose-response experiment was performed in 96-well format using 40,000 cells/well. TR-FRET was measured on RUBYstar instrument (BMG Labtech). Data from a representative experiment are shown.

Typical Product Data – Calcium Assay (Fluorescence)



Agonist	EC_{50} (M)
Neuropeptide S	3.8×10^{-9}

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

Typical Product Data –Whole Cells Radioligand Binding Assay (Filtration)

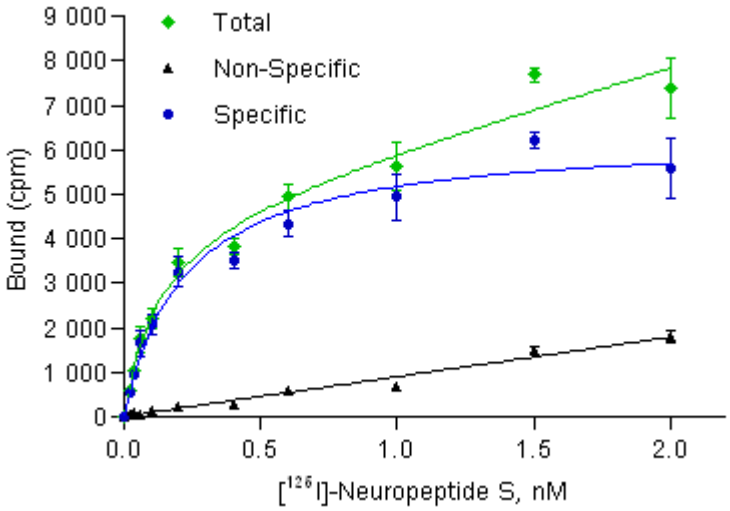
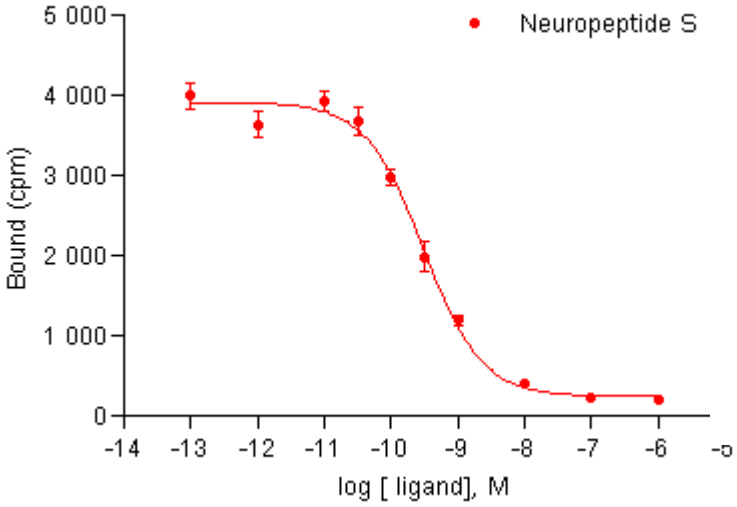


Figure 3: Saturation Binding Assay Curve (Filtration)
A saturation binding assay was performed in 96-well format using 100,000 cells/well. Counts per minute (cpm) were measured on a TopCount® instrument. Data from a representative experiment are shown.



Agonist / Antagonist	IC_{50} (M)
Neuropeptide S	3.1×10^{-10}

Figure 4: Competition Binding Assay Curve (Filtration)
A competition binding assay was performed in 96-well format using 100000 cells /well. Displacement of 0.2 nM [125I]-Neuropeptide S was used. Counts per minute (cpm) were measured on a TopCount® instrument. Data from a representative experiment are shown.

IP-ONE Assay Procedure (TR-FRET)

KREBS solution:	4.2 mM KCl, 146 mM NaCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, 0.1 g/L Glucose, pH 7.4.
Assay Buffer:	KREBS solution + 10 mM LiCl. (provided with the kit).
Cells/well:	40,000. For compounds not tested herein we recommend titrating the cells for optimal performance, i.e. 10,000-80,000 cells per assay point.
Antagonist Pre-incubation:	Simultaneous addition of antagonists with reference agonist.
Agonist Stimulation:	30 min at 37°C

IP1 measurements were performed with the Cisbio International IP-ONE Kit (Cat # 62IP1PEC), according to the manufacturer instructions. Briefly:

1. Compounds were dispensed into a 96-well plate ½ well (Greiner, 675083).
2. Cells in mid-log phase, were detached with PBS-EDTA and seeded in culture medium with 10% FBS (without antibiotics) and incubated overnight at 37°C with 5% CO_2.
3. The next day, culture medium was removed, and 24 µL/well of ligands diluted in Assay Assay Buffer 37°C were added on top of the cells. The plate was incubated for 30 min at 37°C.
4. IP_1 concentrations were then measured following the manufacturer recommendations, and the plate was read with a RUBYstar (BMG Labtech).

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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.

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Whole Cells Radioligand Binding Assay Procedure (Filtration)

Note: For this receptor, we found that apparent receptor expression level was drastically decreased when preparing membranes (which may be indicative of receptor degradation). For this reason, binding was performed using freshly harvested cells.

Assay Buffer:	50 mM Tris-HCl pH 7.4, 0.5% BSA, 0.1 % NaN_3
Wash Buffer:	50 mM Tris-HCl pH 7.4
Radioligand:	[^{125}I]-Neuropeptide S (Revvity # NEX411)
Filters:	Unifilter 96 GF/C (Revvity # 6055690)

Whole Cells Binding Protocol:

Binding assays were performed in 100 µL total volume according to the following conditions. All dilutions are performed in assay buffer:

1. Membrane dilution:	100,000 cells per well (harvested using PBS-EDTA, and resuspended in Assay Buffer), diluted in order to dispense 20 µL/well. Keep on ice.
2. Assembly on ice (in 96 Deep well plate) Saturation Binding:	• 50 µL of assay buffer or of unlabeled ligand (Neuropeptide S, 500 nM final) for determination of non specific binding • 30 µL of radioligand at increasing concentrations (see figure 3) • 20 µL of diluted membranes
Competition Binding:	• 70 µL competitor ligand at increasing concentrations (see figure 4) • 10 µL of radioligand (0.2 nM final) • 20 µL of diluted membranes
3. Incubation:	60 min at 25°C.
4. Filters preparation:	GF/C filters were presoaked in 0.5% PEI at room temperature for 2 hours.
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. Xu YL, Reinscheid RK, Huitron-Resendiz S, Clark SD, Wang Z, Lin SH, Brucher FA, Zeng J, Ly NK, Henriksen SJ, de Lecea L, Civelli O (2004) Neuropeptide S: a neuropeptide promoting arousal and anxiolytic-like effects. Neuron 43:487-497.

2. Reinscheid RK, Xu YL, Okamura N, Zeng J, Chung S, Pai R, Wang Z, Civelli O. (2005) Pharmacological characterization of human and murine neuropeptide s receptor variants. J Pharmacol Exp Ther. 315:1338-1345.

Materials and Instrumentation

The following tables provide the references of compounds and reagents used or recommended for the characterization of the human Neuropeptide S Receptor, isoform A (NPSR1, GPRA) 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
Neuropeptide S (human)	Phoenix Pharmaceuticals	005-89	100 µM in PBS + 0.1% protease-free BSA
[^{125}I]-Neuropeptide S (human)	Revvity	NEX 411	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

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