human Serotonin 5-HT$_{2C}$ (non edited) Receptor Cell Line

Product No.: ES-318-C

Lot No.: 450-441-A

Material Provided

Cells:	2 x 1 mL frozen aliquot (ES-318-CV)
Format:	~2.5 x 10^6 cells /mL in freezing medium

Product Information

Cellular Background:	1321N1
Cell Line Development:	Our proprietary bicistronic expression plasmid containing the sequence coding for the human Serotonin 5-HT$_{2C}$ (non edited) receptor was transfected in 1321N1 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 M81778.1.
Corresponding Protein Sequence:	Identical to GenBank P28335.1: Non edited isoform as described by Saltzman *et al.*, 1991.
Receptor expression level (B_{max}):	Estimated to be 26 pmol/mg protein, using [^{3}H]Mesulergine
K_d for the above radioligand:	0.9 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 B_{max} and K_d for the reference radioligand were determined in saturation binding assay read on a TopCount® instrument. A mycoplasma test was performed using MycoAlert® (Lonza) mycoplasma detection kit. We certify that these results meet our quality release criteria.

[^{3}H] Mesulergine (B_{max}, K_d):	B_{max} = 39 pmoles/mg protein, K_d = 1.8 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 accumulation 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 (1321N1)

- 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:	DMEM, 10% FBS, 1 mM sodium pyruvate, 0.5 mg/ml Geneticin (receptor expression selection).
Freezing Medium:	DMEM, 10% FBS, 1 mM sodium pyruvate 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.

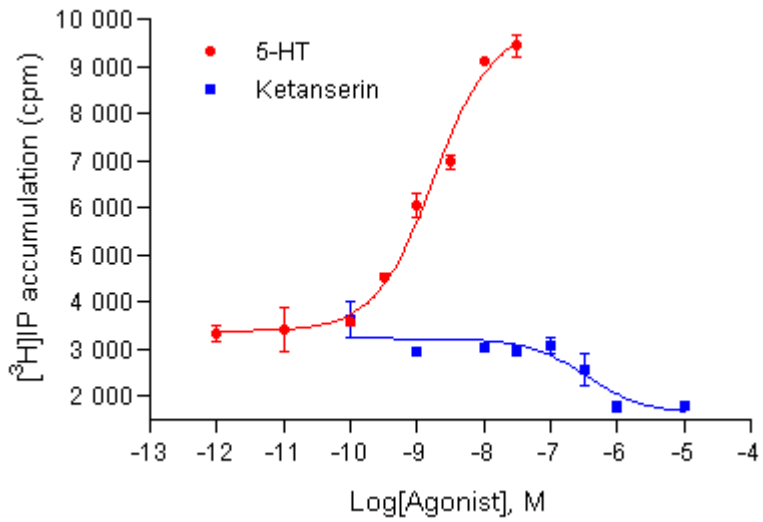
Recommended Seeding Density:	Thawing:	30,000 – 35,000 cells/cm^2
	Log-phase:	19,000 – 23,000 cells/cm^2

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/Inverse Agonist	EC_{50} (M)
5-HT	**1.8×10^{-9}**
Ketanserin	**3.5×10^{-7}**

Figure 1: Agonist / Inverse Agonist Response in Phosphatidylinositol (IP) Accumulation Assay
Cells were collected with PBS-EDTA, loaded with [^{3}H]myoinositol and, after 30 min stimulation of the cells with increasing agonist concentrations, labeled Phosphatidylinositols were separated using chromatography on Dowex columns, as described by Smith *et al.* (1997). Radioactivity, corresponding to the [^{3}H]-labeled Phosphatidylinositol formed was counted with a Tricarb counter. 60,000 cells were used per datapoint. Data from a representative experiment are shown.

Typical Product Data – Inositol Phosphate Assay (SPA)

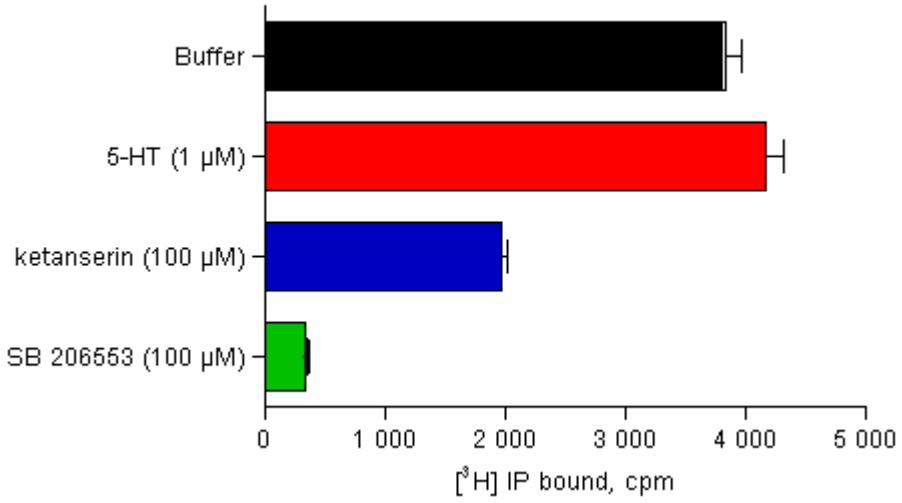


Figure 2. Agonist / Inverse Agonist Response in IP SPA assay
An agonist dose-response experiment was performed in 96-well format using 25,000 cells/well. After loading with [3H] myo-inositol, cells were stimulated with the indicated agonist for 30 min, and then lysed. An aliquot of the cell lysate was 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 –Radioligand Binding Assay (Filtration)

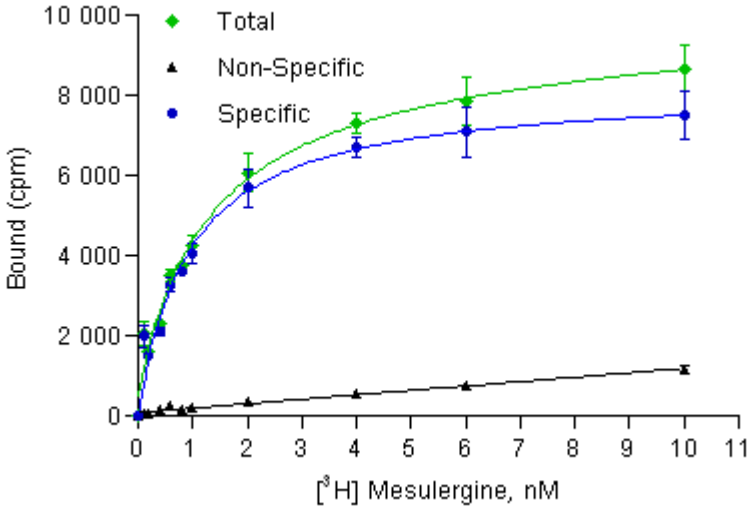
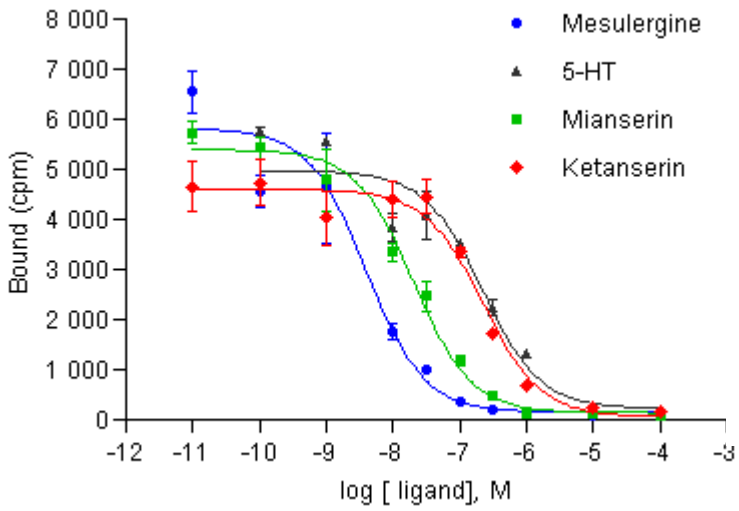


Figure 3: Saturation Binding Assay Curve (Filtration)
A saturation binding assay was performed in 96-well format using 5 µg membranes/well. Counts per minute (cpm) were measured on a TopCount® instrument. Data from a representative experiment are shown.



Agonist / Antagonist	IC_{50} (M)
Mesulergine	**4.0×10^{-9}**
5-HT	**2.2×10^{-7}**
Mianserin	**2.0×10^{-8}**
Ketanserin	**2.2×10^{-7}**

Figure 4: Competition Binding Assay Curve (Filtration)
A competition binding assay was performed in 96-well format using 5 µg membranes/well. Displacement of 0.9 nM [3H]-Mesulergine was used. Counts per minute (cpm) were measured on a TopCount® instrument. Data from a representative experiment are shown.

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Inositol Phosphate Assay Procedure (SPA)

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

Loading Medium: DMEM, w/o L-Glut., w/o i-Inositol (ICN Cat #1642954), 20 µCi/mL Inositol, myo-[2-3H(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 water just before use.

Reader/plate format: TopCount®, 96-well

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 in a cell culture incubator (37°C, 5% CO_2).
Day 2	
3. Cell Loading	At the end of the day, remove the media, wash twice (2 x 100 µL) with inositol-free DMEM and add 100 µL/well of Loading Medium. (keep the remainder of the Loading Medium at 4°C overnight).
4. Incubation	Incubate the assay plate overnight at 37°C in a cell culture incubator.

(Continued)

Day 3	
5. Ligands and compound plates preparation:	Warm at 37°C the remaining Loading Medium (kept overnight at 4°C), Add 10 mM LiCl to an aliquot of Loading Medium 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 Loading Medium pre-heated at 37°C 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 (Revvity 6005290), dispense 90 µL of the 5.55 mg/mL Beads Suspension per well (i.e. 0.5 mg Beads/well) *Note: As beads are sedimenting, it is important to keep beads in suspension when taking them from the stock suspension.* Gently shake the cell plate by inclining it 5 to 10 times, Aspirate 10 µL of cellular lysate (from the bulk of the lysate, avoid touching the cells, avoid 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 more hour without shaking (and up to overnight) before reading the plate and read with a TopCount.
10. Data Analysis:	The cpm measured are used to draw a sigmoidal dose-response curve.

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 pH 7.4, 0.1% ascorbic acid, 4 mM $CaCl_2$

Wash Buffer: 50 mM Tris-HCl pH 7.4

Radioligand: [^{3}H]-Mesulergine (Revvity # NET1148)

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:	4 µg of membranes per well, diluted in order to dispense 500µL/well. Keep on ice.
2. Assembly on ice (in 96 Deep well plate) Saturation Binding:	• 25 µL of assay buffer or of unlabeled ligand (Mianserin, 2 µM final) for determination of non specific binding • 25 µL of radioligand at increasing concentrations (see figure 3) • 500 µL of diluted membranes
Competition Binding:	• 25 µL competitor ligand at increasing concentrations (see figure 4) • 25 µL of radioligand (0.8 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. Smith KE, Forray C, Walker MW, Jones KA, Tamm JA, Bard J, Branchek TA, Linemeyer DL, Gerald C. (1997) Expression cloning of a rat hypothalamic galanin receptor coupled to phosphoinositide turnover. J Biol Chem. 272:24612-24616. *This paper describes the IP accumulation assay procedure.*

2. Saltzman AG, Morse B, Whitman MM, Ivanshchenko Y, Jaye M, Felder S (1991) Cloning of the human serotonin 5-HT2 and 5-HT1C receptor subtypes. Biochem Biophys Res Commun

3. Barker EL, Westphal RS, Schmidt D, Sanders-Bush E (1994) Constitutively active 5-hydroxytryptamine2C receptors reveal novel inverse agonist activity of receptor ligands J Biol Chem

4. Niswender CM, Copeland SC, Herrick-Davis K, Emeson RB, Sanders-Bush E (1999) RNA editing of the human serotonin 5-hydroxytryptamine 2C receptor silences constitutive activity. J Biol Chem

5. Burns CM, Chu H, Rueter SM, Hutchinson LK, Canton H, Sanders-Bush E, Emeson RB (1997) Regulation of serotonin-2C receptor G-protein coupling by RNA editing,. Nature 387:303-308.

6. Filip M, Bader M (2009) Overview on 5-HT receptors and their role in physiology and pathology of the central nervous system. Pharmacol Rep

Materials and Instrumentation

The following tables provide the references of compounds and reagents used or recommended for the characterization of the human Serotonin 5-HT_{2C} (non edited) 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
5-Hydroxytriptamine (5-HT)	Sigma	H9523	10 mM in dH_2O - prepare fresh
Ketanserin	Tocris	0908	10 mM in dH_2O
Mesulergine	Sigma	M153	1 mM in dH_2O
Mianserin	Sigma	M2525	10 mM in ethanol
SB 206553	Sigma	S180	10 mM in PBS + 0.1% BSA
[^{3}H]-Mesulergine	Revvity	NET1148	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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