HUMAN SUBSTANCE P RECEPTOR CELL LINE

Technical Manual No. **Version 20080606**

I. Introduction

Substance P receptor (SPR) is a G protein-coupled receptor (GPCR) that mediates the effects of substance P (SP), a neuropeptide of 11 amino acids. Substance P is believed to be involved in several physiological processes including pain transmission and inflammation. Activation of SPR leads to stimulation of phosphoinositide hydrolysis, stimulation of adenylyl cyclase, and inhibition of an inward rectifying potassium channel. Furthermore, recent studies on rat SPR stably transfected in Chinese hamster ovary (CHO) cells have shown that SPR activation in these cells results in stimulation of phosphoinositide hydrolysis through phospholipase C (PLC), stimulation of AC, and stimulation of arachidonic acid metabolism.

Using HDB's assay development technologies, a cell line and assay protocol was established and pharmacologically validated for SPR responsiveness to the substance P agonist. The substance P receptor assay is ready for high-throughput screening either as a primary or follow-up (selectivity screening) and can be used to identify agonists and antagonists.

II. Cell Line Information

- Description:

  GenScript HUMAN SPR is amplified by PCR using a high-fidelity enzyme and subcloned into the pcDNA3 mammalian expression vector. The full-length ORF has been confirmed by sequencing. The SPR cell line is created by transfection of pcDNA3/SPR in a wt HEK293 cell line. The transfected cells are stably selected by 700μg/ml G418. Single-cell clones with high substance P receptor inducibility are isolated using ring cloning. The clones with the largest dynamic ranges in calcium influx assay are chosen for pharmacological and stability studies.
- Cell Line Name: HEK293/SPR
- Date Created: Nov, 2004
- Function: Cell-based, functional assay for SPR
- Quantity: 1 vial (2×10^6) frozen cells
- Passage Number Shipped: 2
- Host Cell: HEK293
- Cell Phenotype: Adherent/epithelial
- Antibiotics Selection: G418
- Freeze Medium: Growth medium plus 20% FBS and 10% DMSO

- Plasmid: pcDNA3/SPR
- Transfection: Full-length human SPR (also named TACR1) cDNA (Genebank Accession Number: NM_001058)
- Recommended Storage: Liquid nitrogen upon delivery
- Propagation Medium: 90%DMEM, 10% FBS, 700 μg/ml G418, P/E

III. Cell Culture Conditions

Complete Culture Medium:

DMEM: 90%

FBS: 10%

Supplements:

L-glutamine 2.0 mM

Amp 100 μg/ml

Strep 100 μg/ml

Medium for Stable Line Propagation:

Add 700 μg/ml G418 in complete culture medium.

Freezing Medium:

20-90% of fetal bovine serum and 10% dimethyl sulphoxide (DMSO)

Thawing Cells:

1) Quickly thaw frozen cells in a 37°C water bath, agitating continuously.
2) Using a 1 ml pipette, slowly pipet the cells up and down five times and add, drop by drop, to a 15 ml centrifuge tube containing 5 ml of fresh prewarmed complete DMEM medium. Then centrifuge at 1,000 rpm for five minutes.
3) Discard the supernatant medium and resuspend the cell pellet in 5 ml of fresh prewarmed complete DMEM medium. Transfer cells to a T25 flask and incubate at 37°C with 5% CO_2 until the cells reach >90% confluence. The recovery rate for frozen cells is usually 90% or above.

Subculturing:

When the cells reach confluence, they need split. This cell line is normally split twice weekly at 1:8 to 1:15 dilutions.

1) Carefully aspirate all the media. Gently rinse the cell layer with appropriate amount of 0.2% trypsin-EDTA, and aspirate it off.
2) Wait for about 1-3 minutes. Then dislodge the cells by gently tapping the sides of flask or dish.
3) Resuspend cells with appropriate amount of complete DMEM medium, and split cells as desired.

Changing Medium:

This is normally done every other day.

1) Gently aspirate off medium.
2) Transfer fresh warm complete DMEM medium (37°C) into a flask (5 ml for T25 and 10 ml for T75).

Freezing Cells:

1) Repeat the steps 1-3 of subculturing section.

2) Centrifuge down the cells at 1,000 rpm for five minutes.
3) Aspirate off the supernatant and resuspend the cells in fresh freezing medium at a density of 2-3 × 10^6 cells/ml. Add 1 ml cells per cryogenic vial.
4) Put the cryogenic vial of cells into cryo freezing container. Then transfer the container to a -80°C environment and leave it there overnight.
5) Transfer cryogenic vial into liquid nitrogen (-196°C).

IV. Assay Procedure

Calcium Assay of HEK293/SPR Cells

1. 12-24 hours before running the assay, seed the cells in a 96-well black plate at a density of 3 x 10^4 to 5 x 10^4 cells/well.
2. The day of the assay, cells should be 100% confluent. Remove cell plates from the incubator. Do not remove the supernatant. Add an equal volume of Fluo3 Loading Buffer to each well (100 µL per well for 96-well plates, 25 µL per well for 384 well plates).
3. Incubate the plate at 37°C in the dark for one hour.
4. Prepare agonist addition plates in advance of assay. To a 96-well plate, add 10X working concentration of agonist compound in HDB Calcium Assay solution A, and add 25 µl/well to cell plate.
5. Read with FlexStation or FLIPR using the specified settings and save data. The assay should be complete within three to five minutes after addition, but we recommend collecting data for a minimum of 6 minutes during assay development. Usually 90 seconds are enough.

Parameters	
Excitation wavelength (nm)	485
Emission wavelength (nm)	525
Emission cut-off (nm)	515

V. Results

Responses of HEK293/SPR and wt HEK293 Cell Lines to Substance P

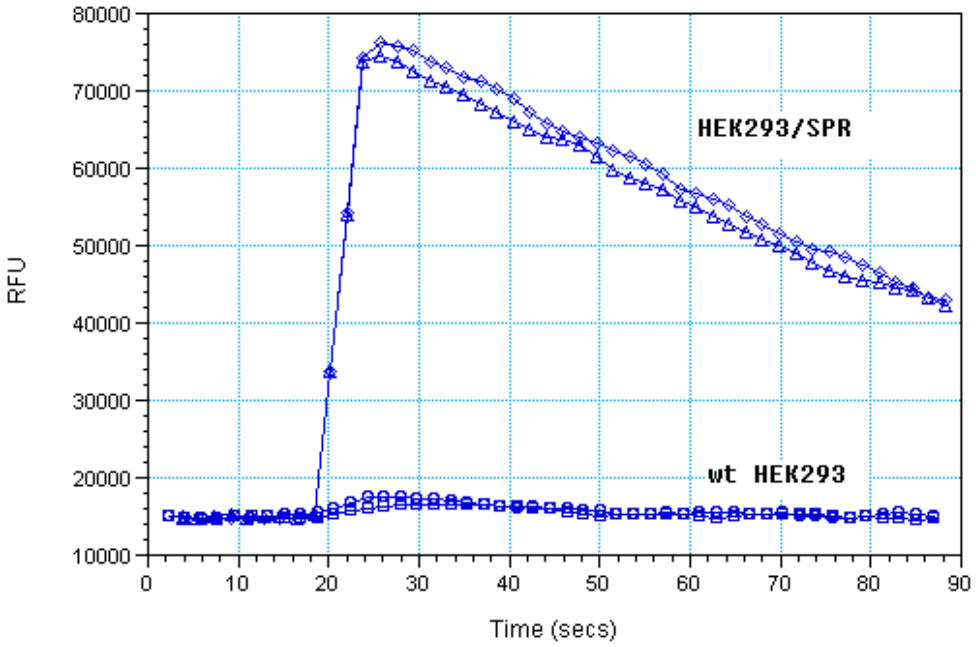


Fig. 1. Responses of HEK293/SPR and wt HEK293 cell lines to Substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH2 synthesized in HD Biosciences). HEK293/SPR cell line showed a significant calcium influx in response to the treatment of 100nM Substance P, while the wt HEK293 cell line did not show apparent response. Assay was monitored with FlexStation plate reader and calcium assay was performed in accordance with the procedure described above.

Substance P Dose Response of HEK293/SPR Cell Line

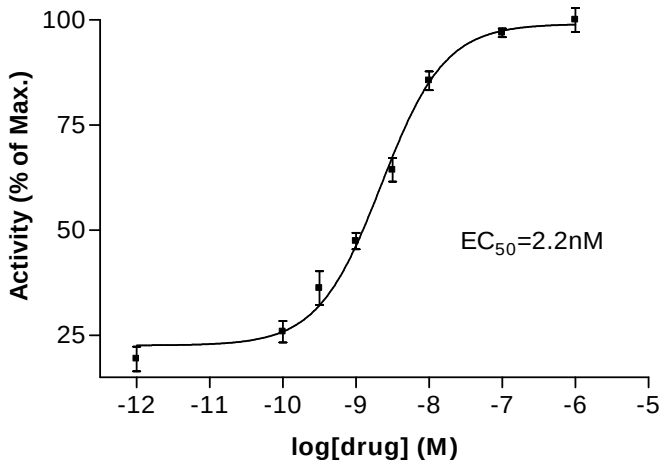


Fig. 2. Dose response of calcium activity as monitored with FlexStation plate reader upon treatment with Substance P. Assay was performed in accordance with the procedure described above. Data represent means ± SEM for duplicate samples. EC50 value for Substance P dose response was determined using GraphPad Prism 4 software.

HEK293/SPR Cell Line Mycoplasma Detection

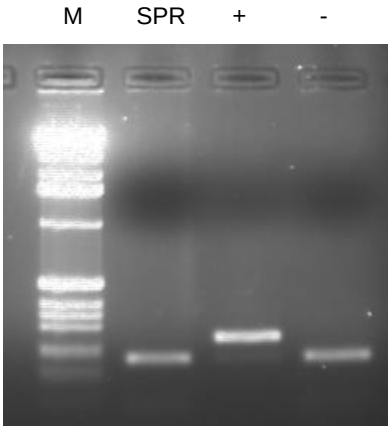


Fig. 3. Mycoplasma detection results with HEK293/SPR cell line. PCR products run on 2% agarose gel.
Lane "SPR" refers to HEK293/SPR cell line.
Lane "+" refers to mycoplasma positive control.
Lane "-" refers to mycoplasma negative control.
Invitrogen 1kb ladder (Cat.No. 15615-016) was used as a marker.

The result demonstrates that cell line is mycoplasma-free.

VI. References

1. Takeda,Y., Chou,K.B., Takeda,J., *et al*. (1991). "Molecular cloning, structural characterization and functional expression of the human substance P receptor. " Biochem. Biophys. Res. Commun. 179 (3), 1232-1240.

2. Bellucci,F., Carini,F., Catalani,C., *et al*. (2002). "Pharmacological profile of the novel mammalian tachykinin, hemokinin 1. " Br. J. Pharmacol. 135 (1), 266-274.

VII. Appendix

Reagents & Consumables:

1. DMEM: Dulbecco's Modified Eagle Medium powder, high glucose (Gibco BRL, Cat #12100-046)
2. FBS: Fetal Bovine Serum (Hyclone, Cat #CH30160.03)
3. L-Glutamine: 200 mM (Gibco BRL, Cat # 25030-081)
4. Ampicillin: 50 mg/ml (Sigma A-9518)
5. Streptomycin Sulfate: 50 mg/ml (Gibco BRL, Cat # 11860-038)
6. G418 Sulfate, cell culture tested. (CALBIOCHEM, Cat #345810)
7. Trypsin: 1:250 rom Bovine Pancreas (Gibco BRL, Cat # 27250016)
8. DMSO: dimethyl sulphoxide, for molecular biology (Sigma, Cat #D8418)
9. Hepes: Sigma Cat #H-3375
10. Fluo-3, AM: (Molecular Probes 28B2-4)
11. Substance P: Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH2 (HD Biosciences)
12. Venor$^{@}$GeM Mycoplasma Detection kit: Minerva Biolabs Cat #11-1050
13. T25 flask: 25 cm^2 cell culture flask (Corning Cat #430639)
14. 6 cm dish: (Orange, Cat # 2050200)
15. 6-well plate: (Corning Cat #3516)
16. Cryogenic Vial: (Corning Cat #430289)
17. 96 Well Plate: Costar, Cat# 3603, Blackwall/clear bottom, Polystyrene, sterilized.

Media and Solutions:

1. PBS (for preparation of 500 ml)

1)	KCl:	0.1 g
2)	KH_2PO_4:	0.1 g
3)	NaCl:	4.0 g
4)	$Na_2HPO_4.12H_2O$:	1.4425 g

Dissolve the above components in double-distilled water (ddH_2O) and adjust pH to 7.4 with 0.1 N NaOH. Add ddH_2O to the final volume of 500 ml. Autoclave and store at 4°C.

2. Trypsin-EDTA (for preparation of 100 ml)

1)	Trypsin:	0.25 g
2)	2%EDTA:	2 ml
3)	PBS:	98 ml

Dissolve trypsin in 2%EDTA and PBS completely. Sterilize the solution by passing through a 0.20 µm membrane filter. Store at 4°C.

3. Culture medium (for preparation of 1 L)

1) Measure out 950 ml distilled water to dissolve the media components, stirring gently until the solution becomes clear.
2) Add $NaHCO_3$ 3.7 g for high glucose DMEM
3) Adjust pH of medium to 0.2-0.3 below the desired final working pH (using 1 N NaOH or 1 N HCL is

recommended). Add slowly while stirring.
4) Dilute to 1 liter with ddH_2O.
5) Sterilize the medium immediately using the method of membrane filtration. Store at 4°C.

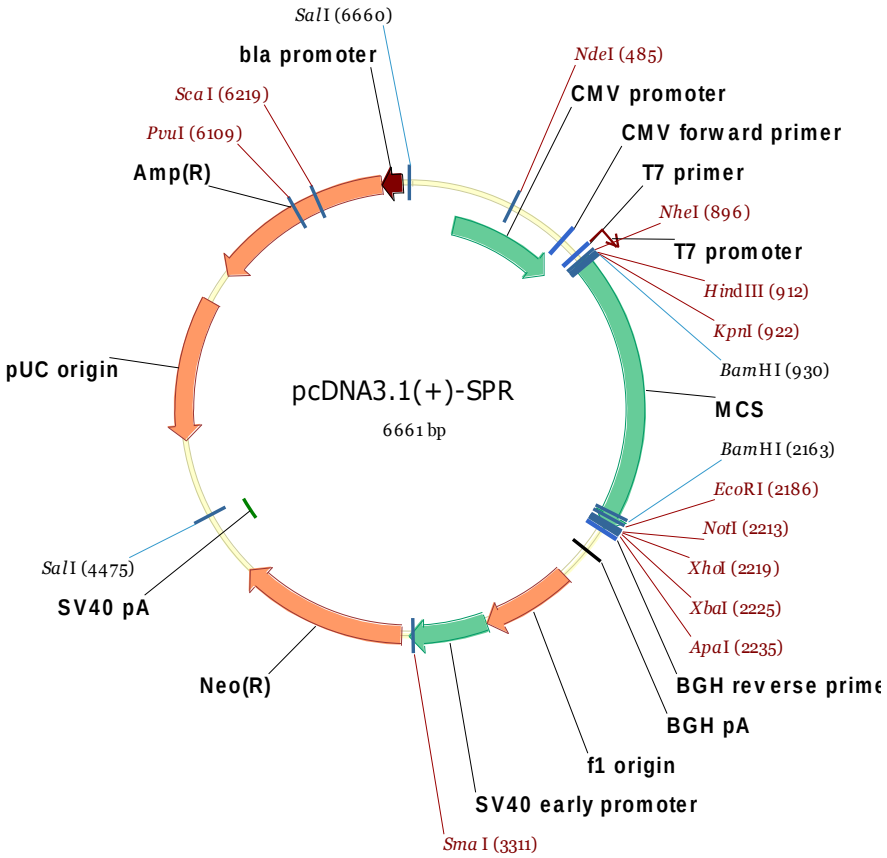
4. Ampicillin/Streptomycin 50 mg/ml

Dissolve 1 g Ampicillin or Streptomycin in 20 ml ddH_2O and sterilize the solution by membrane filtration using 0.20 μm filter. Aliquot and store at 4°C for short-term conservation and -20°C for long-term conservation.

Map of huSPR-pCDNA3.1+

Name: huSPR-pCDNA3.1+
Insert gene: *Homo sapiens* tachykinin receptor 1, (substance P receptor)
Length of insert: 1227 bp Sequence reference: Gene Bank NM_001058
Vector: pCDNA3.1+ (Invitrogen) Insert site: *BamH* I

Plasmid Map:



Comments for huSPR-pCDNA3.1+ 6661 nucleotides

CMV promoter: bases 232-819

T7 promoter/priming site: bases 863-882
huSPR gene: bases 938-2164
pCDNA3.1/BGH reverse priming site: bases 2255-2272
BGH polyadenylation sequence: bases 2261-2485
f1 origin: bases 2531-2959
SV40 early promoter and origin: bases 2964- 3333
Neomycin resistance gene (ORF): bases 3369-4163
SV40 early polyadenylation signal: bases 4337-4467
pUC origin: bases (complementary strand) 4850-5520
Ampicillin resistance gene (*bla*): bases 5665-6525 (complementary strand)

Name: Human tachykinin receptor 1, (substance P receptor) in pCDNA3.1+

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