Human Gonadotropin-Releasing Hormone Receptor Cell Line

Technical Manual No. **Version 20080604**

I. Introduction

The gonadotropin-releasing hormone receptor (GnRHR) is a member of the seven-transmembrane G-protein coupled receptor (GPCR) family. It is expressed on the surface of pituitary gonadotrope cells as well as in the lymphocytes, breast, ovaries, and prostate. Following binding of gonadotropin-releasing hormone, the receptor associates with G-proteins that activate a phosphatidylinositol-calcium second messenger system. Activation of the receptor ultimately causes the release of gonadotropic luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Defects in this gene are a cause of hypogonadotropic hypogonadism (HH).

Using HDB's assay development technologies, a cell line and assay protocol was established and pharmacologically validated for GnRHR responsiveness to the parathyroid hormone receptor agonists and antagonists, GnRH, etc. The GnRHR 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:
  The HUMAN GnRHR is amplified by PCR using a high fidelity enzyme and subcloned into the pcDNA3.1(+) mammalian expression vector. The full-length ORF has been confirmed by sequencing. The GnRHR reporter cell line is created by cotransfection of pcDNA3.1(+)/GNRHR and pNFAT-βla in an Hek293 cell line. The transfected cells are stably selected by 700 μg/ml G418. Single cell clones with high GnRHR inducibility and low β-lactamase background are isolated using ring cloning. The clones with largest dynamic ranges in β-lactamase are chosen for pharmacological and stability studies.
- Cell Line Name: HEK/GnRHR/NFAT/βla
- Date Created: Aug, 2006
- Function: Cell based, functional assay for GnRH receptor
- 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: huGNRHR-pCDNA3.1(+)
- Transfection: Full-length human GnRHR cDNA (Genebank Accession Number: NM_000316)
- Recommended Storage: Liquid nitrogen upon delivery
- Propagation Medium: DMEM, 10% FBS, 700 μg/ml G418

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

Serum-free DMEM:

Same as above but with no FBS
0.1% BSA

Medium for Stable Line Propagation:
Add 700 µg/ml G418 in complete culture medium.

Freezing Medium:
Complete culture medium plus 20% FBS and 10% 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. 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

CCF-4 Assay of HEK293/GnRHR/NFAT/βla Reporter Cells

1. Seed 25,000 cells per well in growth medium (100 µl per well) into 96-well tissue culture treated black-wall, clear-bottom plates (Costar #3603) after trypsinization. Prepare some wells with medium alone (no cells) to use for determining plate background.
2. Culture cells in 5% CO_2 at 37°C. Allow cells to reach ≈90% confluence.
3. 12-24 hours before the assay, replace Growth Medium with 100 µl/well serum-free DMEM. Be careful not to disturb the cells.
4. Prepare ligand solution in serum-free DMEM (10X).
5. Add 10 µl of 10X ligand solution to wells for stimulation and 10 µl of serum-free DMEM per well for non-stimulated control.
6. Incubate cells in 5% CO_2 at 37°C for 5-6 hours.
7. Load cells with 2 µM CCF4/AM as described in CCF4 Loading Protocol.
8. Incubate the plate at room temperature for 60-120 minutes without shaking.
9. Read with Analyst HT plate reader or observe under fluorescence microscope for 60-200 minutes after CCF-4 loading.

CCF-4 Loading Protocol

1. Solution A: Dissolve 5 mg CCF-4/AM with 4.6 ml DMSO to a stock concentration at 1 mM. Aliquot and stored at -80°C, protected from light.
2. Solution B: 100 mg/ml Pluronic -F127 surfactant in DMSO and 0.1% acetic acid
3. Solution C: Red Dye solution
4. 6X substrate loading solution:
   1) Add 6 µl of Solution A to 60 µl of Solution B and mix well
   2) Add 934 µl of Solution C to the combined Solutions A and B and mix well.
5. Add 6X Substrate Loading Solution to cells to 1X final concentration (e.g., add 20 µl of 6XCCF4-AM Substrate Loading Solution to 100 µl of cells in buffer).
6. Add the same volume of 6X Substrate Loading Solution to the cell-free background control wells (containing assay medium or buffer) to 1X final concentration.
7. Cover the plate to protect it from light and evaporation.

V. Results

GnRHR Inducibility of HEK/GnRHR/NFAT/βla Cell Line

	Un-stimulated Control	Stimulated (GnRH)
Ratio 460/530	0.14	3.59
Fold induction	1	25.7

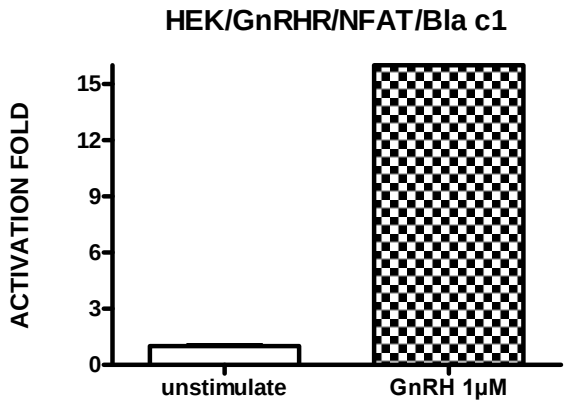


Fig. 1. Bargraph of HEK/GnRHR/NFAT/βla cell line stimulated by GnRH at 1 μM. Assay was done according to the procedures described above.

GnRH Dose Response of HEK/GnRHR/NFAT/βla Cell Line

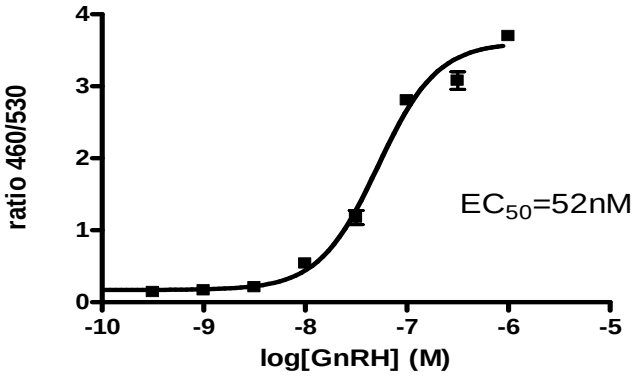


Fig. 2. Dose response of β-lactamase activity as monitored with Analyst HT plate reader upon treatment with ligand. Assay was done according to procedure described above. Data represent means ± SEM for triplicate samples. EC50 value for GnRH dose response was determined using GraphPad Prism 4 software.

HEK/GnRHR/NFAT/βla Cell Line Mycoplasma Detection

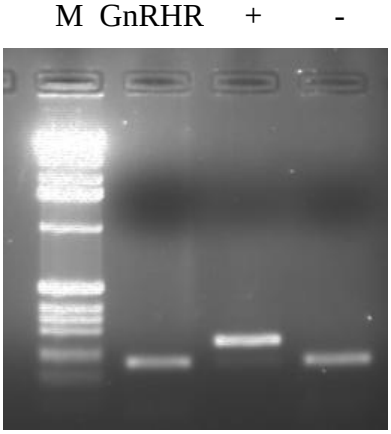


Fig. 3. Mycoplasma detection results with HEK/GnRHR/NFAT/βla cell line. PCR products run on 2% agarose gel.
Lane "GnRHR" referred to HEK/GnRHR/NFAT/βla cell line
Lane "+" referred to Mycoplasma positive control
Lane "-" referred to Mycoplasma negative control
Invitrogen 1kb ladder (Cat.No. 15615-016)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

The result demonstrates that cell line is Mycoplasma free.

VI. References

1. Chi Keung Cheng *et al*. Molecular Biology of Gonadotropin-Releasing Hormone (GnRH)-I, GnRH-II, and Their Receptors in Humans . Endocrine Reviews 26: 283–306, 2005
2. JIMMY D. NEILL *et al*. Minireview: GnRH and GnRH Receptor Genes in the Human Genome. Endocrinology 143: 737–743, 2002
3. Karen L Herbst, Gonadotropin-releasing hormone antagonists. Current Opinion in Pharmacology 3:660–666, 2003

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. Hygromycin B in PBS, 50 mg/ml (Invitrogen, Cat #10687-010)
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. CCF4: (Invitrogen, Cat #K1096)
11. GnRH: synthesized by HD Biosciences
12. Venor$^{@}$GeM Mycoplasma Detection kit: Minerva Biolabs Cat #11-1050
13. T25 flask: 25cm^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 with gentle stirring 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 with 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.

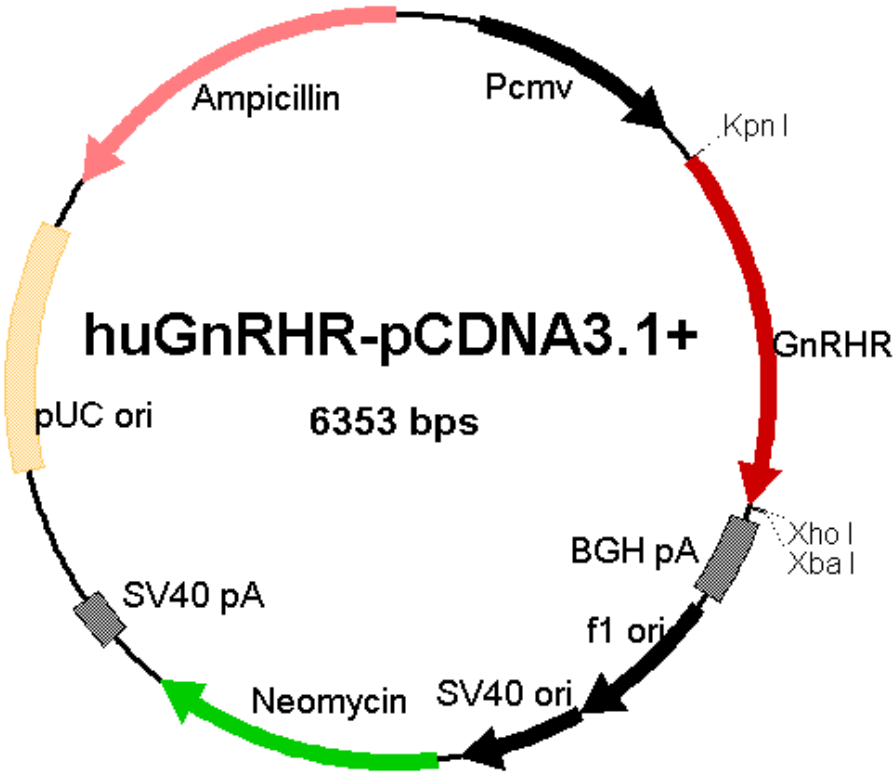
Map of huGnRHR-pCDNA3.1 (+)

Insert gene：gonadotropin-releasing hormone receptor (GnRHR)

Length of insert：987 bp Sequence referance：Gene Bank NM_000406

Vector：pCDNA3.1+ (Invitrogen) Insert site ：*Kpn* I (5') and *Xho*I (3')

Plasmid Map：



Comments for huGnRHR-pCDNA3.1+ 6353 nucleotides

CMV promoter: bases 232~819
T7 promoter/priming site: bases 863~882
GnRHR gene: bases 923 ~1909
pCDNA3.1/BGH reverse priming site: bases 1947~1964
BGH polyadenylation sequence: bases 1953~2177
f1 origin: bases 2223~2651
SV40 early promoter and origin: bases 2656~2999
Neomycin resistance gene (ORF): bases 3061~3855
SV40 early polyadenylation signal: bases 4029~4159
pUC origin: bases (complementary strand) 4542~5212
Ampicillin resistance gene (*bla*): bases 5357~6353(complementary strand)

GenScript Corporation
120 Centennial Ave., Piscataway, NJ 08854
Tel: 732-885-9188, 732-885-9688
Fax: 732-210-0262, 732-885-5878
Email: info@genscript.com
Web: http://www.genscript.com

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