Human Platelet-Activating Factor Receptor Stable Cell Line

Technical Manual No. TM0305 **Version 06042010**

I. Introduction

Platelet-activating factor (PAF) is a bioactive phospholipid that was originally characterized by its ability to induce platelet aggregation but was subsequently shown to mediate angiogenesis, inflammation, and neural development. The human PAF receptor (PAFR) is a member of G protein-coupled receptor family that signals through multiple pathways and mediates several cellular responses including cell motility, smooth muscle contraction, and cytokine and leukotriene release.

This manual describes a cell line and assay protocol established and pharmacologically validated for PAFR responsiveness to the PAFR agonists PAF. The PAFR assay is ready for high-throughput screening either as a primary or follow-up (selectivity screen) and can be used to identify agonists and antagonists.

II. Cell Line Information

- Catalog Number: M00147
- Cell Line Name: HEK293/PAFR/NFAT/β-Lac
- Description:
  The PAFR reporter cell line is created by transfection of pcDNA3.1/hygro(+)PAFR in an HDB parental cell line (Genebank Accession Number: NM_000952), HEK293/NFAT/la. The transfected cells are stably selected by 150 µg/ml hygromycin. Single cell clones with high PAF inducibility and low β-lactamase background are isolated using ring cloning and serial dilution. The clones with largest dynamic range in β-lactamase activity were chosen for pharmacological and stability studies.
- Function: Cell based, functional assay for PAFR receptor
- Quantity: 2 vial (2 x 10^6) frozen cells
- Host Cell: HEK293
- Cell Phenotype: Adherent/epithelial
- Recommended Storage Conditions: Liquid nitrogen (upon delivery)
- Propagation Medium: DMEM, 10% FBS, 150 µg/ml hygromycin, P/E
- Mycoplasma: Negative

III. PAF Dose Response of HEK293/PAFR/NFAT/β-Lac Cell Line and assay protocol

PAF Dose Response On HEK293/PAFR/NFAT/β-Lac cell line

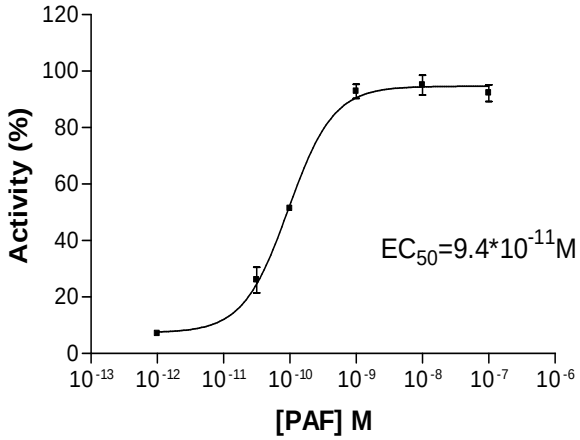


Fig. 1. Dose response of β-lactamase activity as monitored with Analyst HT plate reader upon treatment with PAF. Assay was done according to procedure described as below. Data represent means ± SEM for duplicate samples. EC_{50} value for PAF dose response was determined using GraphPad Prism 4 software (right one).

Assay Procedure

CCF-4 Assay of HEK293/PAFR/NFAT/β-Lac Receptor 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 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 PAF 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 mins without shaking.
9. Read with Analyst HT plate reader or observe under fluorescence microscope for 60-200 mins after CCF-4 loading.

CCF-4 Loading Protocol

1. Solution A: Dissolve 5mg CCF-4/AM with 4.6 ml DMSO to a stock concentration at 1mM. 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 No Cells Background Control wells (containing assay medium or buffer) to 1X final concentration.
7. Cover the plate to protect it from light and evaporation.

IV. References

1. Ferreira, M.A., *et al.*, Sponge-induced angiogenesis and inflammation in PAF receptor-deficient mice (PAFR-KO).Br J Pharmacol, 2004. 141(7): p.1185-1192.
2. Lukashova, V., *et al.*, Janus kinase 2 activation by the platelet-activating factor receptor (PAFR): roles of Tyk2 and PAFR C terminus. J Immunol, 2003. 171(7): p.3794-3800.
3. Yang, W., *et al.*, Chromosomal location, structure, and temporal expression of the platelet-activating factor receptor (PAFr) gene in porcine endometrium and embryos relative to estrogen receptor alpha gene expression. Mol Reprod Dev, 2003. 64(1): p.4-12.
4. Dagenais, P., *et al.*, Modulation of platelet-activating factor receptor (PAFR) gene expression via NF kappa B in MonoMac-1 cells. Inflamm Res, 1997. 46 Suppl 2: p.S161-162.

V. Appendix

Cell Culture Conditions

Note: This clone often grows as clumps. It is necessary to separate cells into single cell suspensions whenever the cells are passaged or plated. MATRIGEL Matrix-coated flasks or plates are recommended for healthier cells.

Complete Culture Medium:
DMEM: 90%, FBS: 10%, penicilin: 100µg /ml, Strep: 100 µg/ml.

Freezing Medium:
45% Complete media, 45% fetal bovine serum and 10% dimethyl sulphoxide (DMSO)

Thawing Cells:

1. Quickly thaw frozen cells in a 37°C water bath with a continuous agitation.
2. Using a 1 ml pipette, slowly pipet the cells up and down 5 times and add, drop by drop, to a 15 ml centrifuge tube containing 9 ml of fresh pre-warmed complete DMEM medium. Then centrifuge at 1,000 rpm for 5 minutes.
3. Discard the supernatant medium and resuspend the cell pellet in 5 ml of fresh prewarmed complete DMEM medium. Transfer cells to a 10cm dish 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 0.05% trypsin-EDTA, and aspirate it off.
2. Wait for about 1-3 minute; 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 10cm dish (10 ml for one 10cm dish).

Freezing Cells:

1. Repeat the steps 1-3 of Subculturing section.
2. Centrifuge down the cells at 1,000 rpm for 5 min.
3. Aspirate off the supernatant and resuspend the cells in fresh freezing medium at a density of 2-3 x 10^6 cells/ml. Add 1 ml cells per Cryogenic Vial.
4. Put the Cryogenic Vial of cells into Cryo Freezing Container, followed by transferring the container into -80°C and staying overnight.

Transfer Cryogenic Vial into liquid nitrogen (-196°C).

Reagents & Consumables:

1. DMEM: Dulbecco's Modified Eagle Medium powder, high glucose (Gibco BRL, Cat #10566)
2. FBS: Fetal Bovine Serum (GIBCO, Cat #26140)
3. Streptomycin Sulfate: 50 mg/ml (Gibco BRL, Cat # 11860-038)
4. Hygromycin (Invitrogen, Cat #379685)
5. Trypsin: (Gibco BRL, Cat # 25300)
6. DMSO: dimethyl sulphoxide, for molecular biology (AMRESCO, Cat #0231)
7. CCF4: (Invitrogen, Cat #K1096)
8. Venor$^{@}$GeM Mycoplasma Detection kit: Minerva Biolabs Cat #11-1050
9. 10cm dish: Corning Cat #430167)
10. 6-well plate: (Costar Cat #3516)
11. Cryogenic Vial: (Corning Cat #430659)
12. 96 Well Plate: greiner, Cat# 655180
13. 384 Well Plate: Corning Cat #3712

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) $Na_2HPO_4.12H_2O$: 1.4 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 room temperature.

2. 2. Trypsin-EDTA (for preparation of 100 ml)
   1) 0.5%Trypsin: 10 ml
   2) DMEM: 90 ml

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 hPAFR-pcDNA3.1/hygro(+)

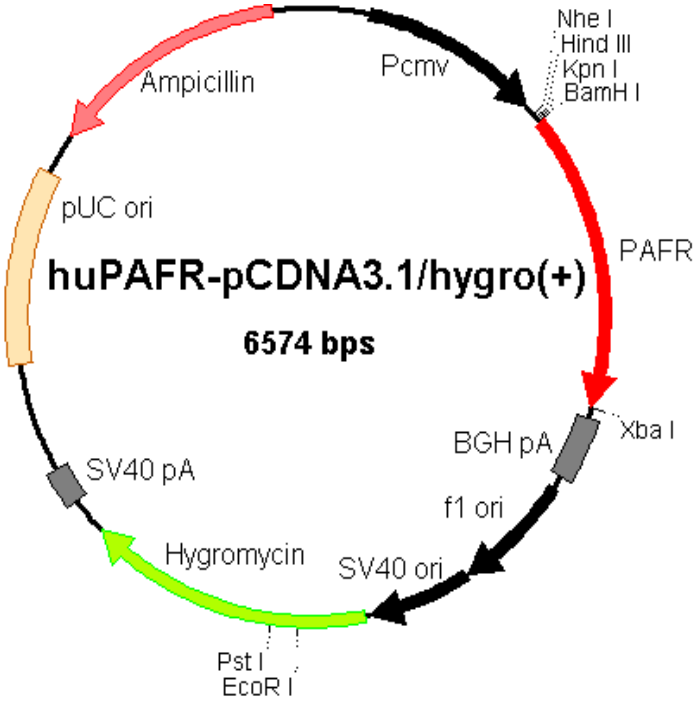
Name：huPAFR-pCDNA3.1/hygro(+)

Insert gene: Homo sapiens platelet-activating factor receptor (PAFR)

Length of insert： 1029 bp Sequence reference: Gene Bank NM_000952

Vector：pCDNA3.1/hygro(+) (Invitrogen) Insert site ：*BamH* I + *Xba* I

Plasmid Map：



Comments for huPAFR-pCDNA3.1/hygro(+) 6574 nucleotides

CMV promoter: bases 209~863
T7 promoter/priming site: bases 863~882
PAFR gene: bases 935~1963
pCDNA3.1/BGH reverse priming site: bases 1995~2012
BGH polyadenylation signal: bases 2001~2225
f1 origin: bases 2271~2699
SV40 early promoter and origin: bases 2704~3074
Hygromycin resistance gene (ORF): bases 3092~4117
SV40 early polyadenylation signal: bases 4247~4377
pUC origin: bases 4760~5433 (complementary strand)
Ampicillin resistance gene (*bla*): bases 5578~6438 (complementary strand)

Name：Human platelet-activating factor receptor (PAFR) in pCDNA3.1/hygro(+)

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