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PRODUCT DATASHEET

ChemiBrite™ EP_4 Prostanoid Receptor Stable Cell Line

CATALOG NUMBER: HTS142L

CONTENTS: 2 vials of mycoplasma-free cells, 1 mL per vial.

STORAGE: Vials are to be stored in liquid N_2.

BACKGROUND

ChemiBrite cells express a novel variant of clytin, a calcium-activated photoprotein, to enable sensitive luminescent detection of ligand-induced calcium flux. The ChemiBrite version of clytin contains a mutation that increases its affinity for calcium to a level that permits detection of cytosolic calcium in many cells with greater sensitivity than other mitochondrially expressed photoproteins. Luminescent calcium assays offer several advantages over fluorescent calcium assays including; increased sensitivity and lack of interference from fluorescent compounds.

Prostanoids are a series of arachidonic acid metabolites produced by the action of cyclooxygenase and further modified by isomerases and synthases. Cells rapidly secrete prostanoids after synthesis, whereupon the prostanoids bind to a family of 8 GPCRs to exert their biological effects (Narumiya and FitzGerald, 2001). The prostaglandin PGE2 causes pain, vasodilation, immunosuppression of T cells, bone remodeling and promotion of carcinogenesis. Four related GPCRs: EP1, EP2, EP3 and EP4, each bind to PGE2, but the different G protein coupling status of each receptor leads to distinct biological effects. EP_4 couples primarily to Gs to increase intracellular cAMP levels. During neonatal development, EP_4 participates in closure of the ductus arteriosus, a process required for switching circulation from the placenta to the lungs (Nguyen et al., 1997). In addition, EP_4 mediates PGE2-induced bone formation by promoting osteoblastogenesis, and selective EP_4 agonists are being evaluated as potential treatments for osteoporosis (Yoshida et al., 2002). Millipore's cloned EP_4 receptor-expressing ChemiBrite cells are made by stable transfection of HEK293 cells with ChemiBrite clytin, EP_4 receptor and a promiscuous G protein to couple the receptor to the calcium signaling pathway. These stability tested cells are ready for luminescent analysis of agonists, antagonists and modulators at the EP_4.

USE RESTRICTIONS

Please see **Limited Use Label License Agreement** (Label License Agreement) for further details.

WARNINGS

For Research Use Only; Not for Use in Diagnostic Procedures
Not for Animal or Human Consumption

GMO

This product contains genetically modified organisms.
Este producto contiene organismos genéticamente modificados.
Questo prodotto contiene degli organismi geneticamente modificati.
Dieses Produkt enthält genetisch modifizierte Organismen.
Ce produit contient organismes génétiquement des modifiés.
Dit product bevat genetisch gewijzigde organismen.
Tämä tuote sisältää geneettisesti muutettuja organismeja.
Denna produkt innehåller genetiskt ändrade organismer.

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APPLICATIONS

Calcium Flux Assays : Luminescent Mode and Fluorescent Mode ; cAMP Accumulation

APPLICATION DATA

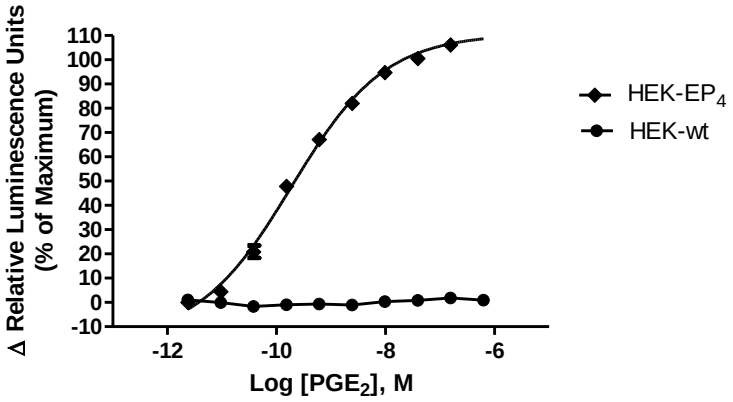

Figure 1. Representative data for activation of EP_4 receptor stably expressed in HEK293 cells induced by PGE_2 using a luminescent calcium flux assay. EP_4–expressing HEK293 cells were loaded with 10 µM coelenterazine for 3 h at room temperature and calcium flux in response to the indicated ligand was determined on a Molecular Devices FLIPR^{TETRA}® with ICCD camera in 96-well format with a final concentration of 0.5% DMSO. Luminescence signal obtained in this experiment was 240,000 RLU (Relative Light Units) as measured by AUC (are under curve) for 80 s post agonist addition using the provided protocol. Similarly parental cells (catalog #: HTSHEK-2L) were tested to determine the specificity of the resulting signal.

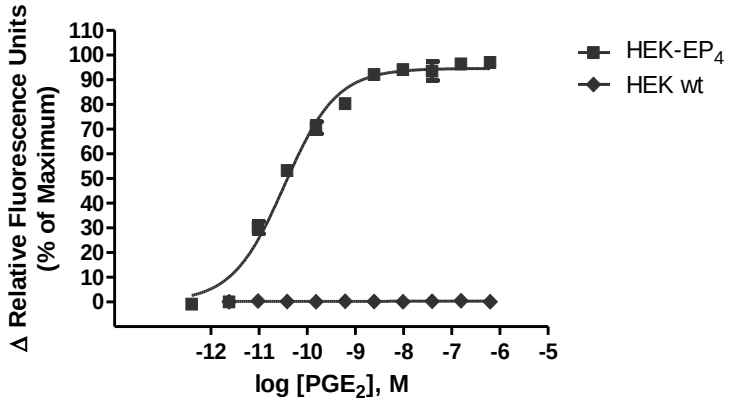

Figure 2. Representative data for activation of EP_4 receptor stably expressed in HEK293 cells induced by PGE2 using a fluorescent calcium flux assay. EP_4–expressing HEK293 cells were seeded at 50,000 cells per well into a 96-well plate, the following day the cells were loaded with a calcium dye, and calcium flux in response to the indicated ligand with a final concentration of 0.5% DMSO was determined on a Molecular Devices FLIPRTETRA® with ICCD camera. Maximal fluorescence signal obtained in this experiment was 18,000 RFU. Similarly parental cells (catalog #: HTSHEK-2L) were tested to determine the specific of the resulting signal.

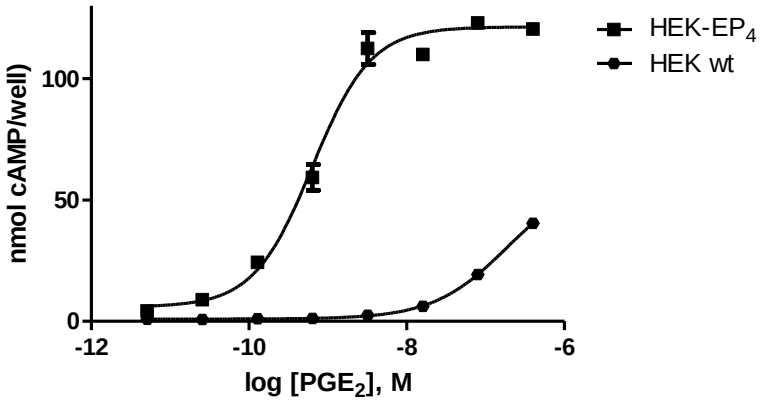

Figure 3. Representative data for activation of EP_4 receptor stably expressed in HEK293 cells induced by PGE_2 using a cAMP accumulation assay. EP_4–expressing HEK293 cells were seeded at 25,000 cells per well into a 96-well plate. The following day the cells were treated with PGE_2 for 10 minutes in the presence of 2 mM IBMX and 0.5% DMSO to determine receptor mediated cAMP generation using a time-resolved fluorescence resonance energy transfer (TR-FRET) assay measured on the BioTek Synergy. Similarly parental cells (catalog #: HTSHEK-2L) were tested to determine the specificity of the resulting signal.

Table 1. EC_{50} values of EP_4-expressing HEK293 cells.

LIGAND	ASSAY	POTENCY EC_{50} (nM)	REFERENCE
Prostaglandin E2	Calcium Flux - Fluorescence	0.3*	Eurofins Internal Data
Prostaglandin E2	Calcium Flux - Luminescence	0.03	Eurofins Internal Data
Prostaglandin E2	cAMP Accumulation	0.5	Eurofins Internal Data

* The cell line was tested and found to have equivalent EC_{50} and signal at 1, 3 and 6 weeks of continuous culture by calcium flux fluorescence. The Z' value, as defined with response to 10µM PGE2, was 0.8.

CELL CULTURE

Table 2. Recommended Cell Culture Reagents (not provided)

Description	Component	Concentration	Supplier and Product Number
Basal Medium	DMEM F12	-	Millipore: DF041-B
	Fetal Bovine Serum (FBS)	10%	Hyclone: SH30070.03
	Non-Essential Amino Acids (NEAA)	1X	Hyclone: SH30238.01
Selection Medium	Basal Medium (see above)	-	
	Geneticin (G418)	400 µg/ml	Invivogen: ant-gn-5
	Puromycin	1 µg/ml	Merck: EMD 4005[illegible]3
	Hygromycin	200 µg/ml	Invivogen: ant-hg-5
Dissociation	Sterile PBS	-	Hyclone: SH30028.03
	0.05% Trypsin-EDTA	-	Hyclone: SH30042.01
CryoMedium	Basal Medium (see above)	40%	
	Fetal Bovine Serum (FBS)	50%	Hyclone: SH30070.03
	Dimethyl Sulfoxide (DMSO)	10%	Sigma: D2650

Cell handling

1. Upon receipt, directly place cells in liquid nitrogen storage. Consistent cryopreservation is essential for culture integrity.
2. Prepare Basal Medium. Prepare 37ºC Water Bath. Thaw cells rapidly by removing from liquid nitrogen, and immediately immersing in a 37ºC water bath, until 90% thawed. Immediately sterilize the exterior of the vial with 70% ethanol.
3. Add vial contents to 15 mL Basal Medium in T75 Tissue Culture Treated Flask. Gently swirl flask and place in a humidified, tissue culture incubator, 37ºC, 5% CO_2.
4. 18-24 Hours Post–Thaw, all live cells should be attached. Viability of the cells is expected to be 60-90%, At this time, exchange Basal Medium with Selection Medium.
5. When cells are approximately 80% confluent, passage the cells. It is suggested that user expand culture to create >20 vial Master Cell Bank at low passage number. *Cells should be maintained at less than 80% confluency for optimal assay results.*
6. Cell Dissociation: Aspirate Culture Medium. Gently wash with 1x Volume PBS. Add 0.1x Volume Warm Trypsin-EDTA. Incubate 4 min, 37°C, until cells dislodge. *If cells do not round up, place in 37º C incubator for additional 2 min*. Neutralize Trypsin and collect cells in 1x Volume Basal Medium.
7. Seed Cells for expansion of culture. It is recommended that cell lines are passaged at least once before use in assays.

Table 3. Cell Culture Seeding Suggestions: *User should define based on research needs.*

Flask Size (cm^2)	Volume (mL)	Total Cell Number ($x10^6$)	Growth Period (hrs)
T75	15	2.0	24
T75	15	1.0	48
T75	15	0.8	72
T150	30	4.0	24
T150	30	3.0	48
T150	30	2.0	72

ASSAY SETUP

Luminescence

Table 4. Settings for FLIPR^{TETRA}® with ICCD camera option

Option	Setting
Read Mode	Luminescence
Ex/Em	None/None
Camera Gain	280,000
Gate Open	100 %
Exposure Time	0.9 sec
Read Interval	1 sec.
Dispense Volume	50 µl (25 µl for 384-well)
Dispense Height	95 µl (50 µl for 384-well)
Dispense Speed	50 µl/sec
Expel Volume	0 µl
Analysis	Subtract Bias Sample 1

Table 5. Luminescence Assay Materials (Not provided)

Description	Supplier and Product Number
HBSS	Invitrogen: 14025
HEPES 1M Stock	Millipore: TMS-003-C
BSA (Protease Free). Prepare to 1% in H_2O, filter	Merck EMD: 126609
Prostaglandin E2 ligand	Cayman: 14010
Non-Binding 96/384 well Plates (for ligand prep)	Corning: 3605/ 3574
Black (clear Bottom) cell assay plates	Corning: 3904/ 3712
Coelenterazine (250ug). Prepare to 10mM	Merck EMD: 233900

Assay Protocol – Luminescence

1. Dissociate Culture as Recommended. Collect in Basal Medium. Document Cell Count and Viability
2. Centrifuge the cell suspension at 190 x g for six min
3. Remove supernatant. Gently resuspend the cell pellet in Basal Medium. *It is suggested that end user optimize cell plating based on individual formats.* (Default: Resuspend in volume to achieve 5x10^5cells/ml *(i.e, if collected 5e6 TC,* $^{5e6/}_{5e5/ml}$ *=10 mL volume)*
4. Seed cell suspension into black, clear bottom plate (100 µL/well for 96-well plate). *When seeding is complete, place the assay plate at room temperature for 30 min.*
5. Move assay plate to a humidified 37ºC 5% CO_2 incubator for 18-24 h.
6. Next day, prepare Assay buffer (HBSS, 20mM HEPES, pH 7.4) and Loading buffer (Assay buffer with 10µM coelenterazine). *Note: Please prepare coelenterazine stock according to Manufacturer's Recommendations at 10mM to allow for 1:1000 dilution into Loading buffer (10uM final concentration). It is critical that coelenterazine solution is prepared at room temperature and is protected from light.*

7. Remove medium from assay plate.
8. Add Loading buffer to assay plate (100 µL/well for 96-well plate). Incubate plate for 1.5 h at room temperature, protected from light.
9. Prepare ligands in assay buffer at 3x final concentration in non-binding plates. Use Buffer Only Control Wells for Background Subtraction.
10. Create protocol for ligand addition. Please refer to FLIPR^{TETRA}® settings provided in Table 2. Set time course for 180 s, with ligand addition at 10 s.
11. After the run is complete, apply subtract bias on sample 1. Export data to analyze using the area under the curve statistic.

Fluorescence

FLIPR SETTINGS

Table 6. Settings for FLIPR^{TETRA}® with ICCD camera option

Option	Setting
Read Mode	Fluorescence
Ex/Em	Ex470_495 / Em515_575
Camera Gain	2000
Gate Open	6 %
Exposure Time	0.53
Read Interval	1s
Dispense Volume	50 µl (25 µl for 384-well)
Dispense Height	25 µl (50 µl for 384-well)
Dispense Speed	75 µl L/sec (50 µl for 384-well)
Expel Volume	0 µl
Analysis	Subtract Bias Sample 1

Table 7. Fluorescence Assay Materials (Not provided)

Description	Supplier and Product Number
HBSS	Hyclone: SH30268.02
HEPES 1M Stock	EMD Millipore.: TMS-003-C
Probenicid	Sigma: P8761
Quest Fluo-8™, AM	AAT Bioquest: 21080
PDE_2 ligand	Cayman: 14010
Non-binding white plates (for ligand prep)	Corning: 3605(96-well)/3574(384-well)
Black (clear bottom) tissue-culture treated plates	Corning: 3904(96-well)/3712(384-well)

Assay Protocol – Fluorescence

8. Immediately upon receipt, thaw cells or place cells in liquid nitrogen.
9. Thaw cells rapidly by removing from liquid nitrogen and immediately immersing in a 37ºC water bath. Immediately after ice has thawed, sterilize the exterior of the vial with 70% ethanol.
10. Add 1mL of pre-warmed Media Component to each vial of cells. Place contents from two vials into a 15 mL conical tube and bring the volume to 10 mL of Media Component.

11.	Centrifuge the cell suspension at 190 x g for four minutes
12.	Remove supernatant and add 10.5 mL of pre-warmed Media Component to resuspend the cell pellet.
13.	Seed cell suspension into appropriate assay microplate (100 µL/well for 96-well plate, 25 µL/well for 384-well plate).
14.	When seeding is complete, place the assay plate at room temperature for 30 minutes.
15.	Move assay plate to a humidified 37ºC 5% CO2 incubator for 24 hours.
16.	After 24 hour incubation, remove assay plate from the incubator and invert plate to remove Media Component.
17.	Prepare Fluo-8, AM (AAT Bioquest: 21080) Ca^{2+} dye by dissolving 1mg of Fluo-8 NW in 200 µL of DMSO. Once dissolved place 10 µL of Fluo-8 NW Ca^{2+} dye solution into 10 mL of HBSS 20mM HEPES, 2.5mM Probenecid pH 7.4 buffer and apply to assay microplate (Ca^{2+} dye at 10 µL /10 mL is sufficient for loading one (1) microplate).
18.	Set-up FLIPR to dispense 3x ligand to appropriate wells in the assay plate. Set excitation wavelength at 470-495 nm ($FLIPR^{TETRA}$) or 485 nm (FLIPR1, FLIPR2, FLIPR3) and emission wavelength at 515-565 nm ($FLIPR^{TETRA}$) or emission filter for Ca^{2+} dyes (FLIPR1, FLIPR2, FLIPR3). Set pipet tip height to 5 µL below liquid level and dispense rate to 75 µL/sec (96-well format) or 50 µL/sec (384-well format). Set up plate layout and tip layout for each individual experiment. Set time course for 180 seconds, with ligand addition at 10 seconds.
19.	Ligands are prepared in non-binding surface Corning plates (Corning 3605 – 96-well or Corning 3574 – 384-well).
20.	After the run is complete, negative control correction is applied and data analyzed utilizing the maximum statistic.

HOST CELL

HEK293

EXOGENOUS GENE EXPRESSION

Human PTGER4 cDNA (Accession Number: NM000958; see CODING SEQUENCE below) and a proprietary mutant clytin photoprotein, and promiscuous G protein, each expressed in a bicistronic vector

CODING SEQUENCE

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ATG TCC ACT CCC GGG GTC AAT TCG TCC GCC TCC TTG AGC CCC GAC CGG CTG AAC AGC CCA GTG ACC ATC CCG
 M   S   T   P   G   V   N   S   S   A   S   L   S   P   D   R   L   N   S   P   V   T   I   P

GCG GTG ATG TTC ATC TTC GGG GTG GTG GGC AAC CTG GTG GCC ATC GTG GTG CTG TGC AAG TCG CGC AAG GAG
A   V   M   F   I   F   G   V   V   G   N   L   V   A   I   V   V   L   C   K   S   R   K   E

CAG AAG GAG ACG ACC TTC TAC ACG CTG GTA TGT GGG CTG GCT GTC ACC GAC CTG TTG GGC ACT TTG TTG GTG
Q   K   E   T   T   F   Y   T   L   V   C   G   L   A   V   T   D   L   L   G   T   L   L   V

AGC CCG GTG ACC ATC GCC ACG TAC ATG AAG GGC CAA TGG CCC GGG GGC CAG CCG CTG TGC GAG TAC AGC ACC
S   P   V   T   I   A   T   Y   M   K   G   Q   W   P   G   G   Q   P   L   C   E   Y   S   T

TTC ATT CTG CTC TTC TTC AGC CTG TCC GGC CTC AGC ATC ATC TGC GCC ATG AGT GTC GAG CGC TAC CTG GCC
F   I   L   L   F   F   S   L   S   G   L   S   I   I   C   A   M   S   V   E   R   Y   L   A

ATC AAC CAT GCC TAT TTC TAC AGC CAC TAC GTG GAC AAG CGA TTG GCG GGC CTC ACG CTC TTT GCA GTC TAT
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 I   N   H   A   Y   F   Y   S   H   Y   V   D   K   R   L   A   G   L   T   L   F   A   V   Y

GCG TCC AAC GTG CTC TTT TGC GCG CTG CCC AAC ATG GGT CTC GGT AGC TCG CGG CTG CAG TAC CCA GAC ACC
 A   S   N   V   L   F   C   A   L   P   N   M   G   L   G   S   S   R   L   Q   Y   P   D   T

TGG TGC TTC ATC GAC TGG ACC ACC AAC GTG ACG GCG CAC GCC GCC TAC TCC TAC ATG TAC GCG GGC TTC AGC
 W   C   F   I   D   W   T   T   N   V   T   A   H   A   A   Y   S   Y   M   Y   A   G   F   S

TCC TTC CTC ATT CTC GCC ACC GTC CTC TGC AAC GTG CTT GTG TGC GGC GCG CTG CTC CGC ATG CAC CGC CAG
 S   F   L   I   L   A   T   V   L   C   N   V   L   V   C   G   A   L   L   R   M   H   R   Q

TTC ATG CGC CGC ACC TCG CTG GGC ACC GAG CAG CAC CAC GCG GCC GCG GCC GCC TCG GTT GCC TCC CGG GGC
 F   M   R   R   T   S   L   G   T   E   Q   H   H   A   A   A   A   S   V   A   S   R   G

CAC CCC GCT GCC TCC CCA GCC TTG CCG CGC CTC AGC GAC TTT CGG CGC CGC CGG AGC TTC CGC CGC ATC GCG
 H   P   A   A   S   P   A   L   P   R   L   S   D   F   R   R   R   R   S   F   R   R   I   A

GGC GCC GAG ATC CAG ATG GTC ATC TTA CTC ATT GCC ACC TCC CTG GTG GTG CTC ATC TGC TCC ATC CCG CTC
 G   A   E   I   Q   M   V   I   L   L   I   A   T   S   L   V   V   L   I   C   S   I   P   L

GTG GTG CGA GTA TTC GTC AAC CAG TTA TAT CAG CCA AGT TTG GAG CGA GAA GTC AGT AAA AAT CCA GAT TTG
 V   V   R   V   F   V   N   Q   L   Y   Q   P   S   L   E   R   E   V   S   K   N   P   D     L

CAG GCC ATC CGA ATT GCT TCT GTG AAC CCC ATC CTA GAC CCC TGG ATA TAT ATC CTC CTG AGA AAG ACA GTG
 Q   A   I   R   I   A   S   V   N   P   I   L   D   P   W   I   Y   I   L   L   R   K   T   V

CTC AGT AAA GCA ATA GAG AAG ATC AAA TGC CTC TTC TGC CGC ATT GGC GGG TCC CGC AGG GAG CGC TCC GGA
 L   S   K   A   I   E   K   I   K   C   L   F   C   R   I   G   G   S   R   R   E   R   S   G

CAG CAC TGC TCA GAC AGT CAA AGG ACA TCT TCT GCC ATG TCA GGC CAC TCT CGC TCC TTC ATC TCC CGG GAG
 Q   H   C   S   D   S   Q   R   T   S   S   A   M   S   G   H   S   R   S   F   I   S   R   E

CTG AAG GAG ATC AGC AGT ACA TCT CAG ACC CTC CTG CCA GAC CTC TCA CTG CCA GAC CTC AGT GAA AAT GGC
 L   K   E   I   S   S   T   S   Q   T   L   L   P   D   L   S   L   P   D   L   S   E   N   G

CTT GGA GGC AGG AAT TTG CTT CCA GGT GTG CCT GGC ATG GGC CTG GCC CAG GAA GAC ACC ACC TCA CTG AGG
 L   G   G   R   N   L   L   P   G   V   P   G   M   G   L   A   Q   E   D   T   T   S   L   R

ACT TTG CGA ATA TCA GAG ACC TCA GAC TCT TCA CAG GGT CAG GAC TCA GAG AGT GTC TTA CTG GTG GAT GAG
 T   L   R   I   S   E   T   S   D   S   S   Q   G   Q   D   S   E   S   V   L   L   V   D   E

GCT GGT GGG AGC GGC AGG GCT GGG CCT GCC CCT AAG GGG AGC TCC CTG CAA GTC ACA TTT CCC AGT GAA ACA
 A   G   G   S   G   R   A   G   P   A   P   K   G   S   S   L   Q   V   T   F   P   S   E   T

CTG AAC TTA TCA GAA AAA TGT ATA TGA
 L   N   L   S   E   K   C   I  Stp
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RELATED PRODUCTS

PRODUCT NUMBER	DESCRIPTION
HTSHEK-2L	ChemiBrite™ HEK293 stable parental cell line
HTS142M	ChemiScreen™ EP_4 Prostanoid Receptor membrane prep
HTS142RTA	Ready-to-Assay™ EP_4 Prostanoid Receptor frozen cells
HTSCHEM-1RTA	Ready-to-Assay™ Chem-1 host frozen cells (control cells)

REFERENCES

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3. Yoshida K *et al.* (2002) Stimulation of bone formation and prevention of bone loss by prostaglandin E EP4 receptor activation. *Proc. Natl. Acad. Sci. USA* 99: 4580-5.
4. Wilson R *et al* (2004) Functional Pharmacology of human prostanoid EP_2 and EP_4 Receptors, *Eur J Pharmacology* 501: 49-58

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