TECHNICAL DATA SHEET

PhotoScreen® Ion Channel Cell Line

Caution: For Laboratory Use. A product for research purposes only

Human Purinergic P2RX4 Ion Channel

Product No.: AX-015-PCF

Lot No.: 1840458

Material Provided

Cells:	2 x 1 mL frozen aliquot (AX-015-PCFV)
Format:	~2.5 x 10^6 cells/mL in complete medium with 10% DMSO

Product Information

Cellular Background:	HEK-293
Cell Line Development:	HEK-293 cells stably expressing mitochondrially targeted Photina® photoprotein were transfected with a pCDNA3.1 expression vector containing the coding sequence of the human P2RX4 ion channel under the control of the CMV promoter. Resistant clones were obtained by limiting dilution and compared for their response to a reference agonist using the PhotoScreen® assay. The selected clone has also been functionally validated by electrophysiology experiments.
GenBank Accession Number:	Identical to coding sequence of NM_002560.2 (DNA) and NP_002551.2 (protein)
Storage Conditions:	Store in liquid nitrogen.
Membrane Receptor (B_{MAX}):	Not determined for this cell line.

Quality Control

EC_{50} for a reference agonist is determined using a PhotoScreen® assay performed with adherent cells on the LumiLux®. Mycoplasma test is performed using MycoAlert® Mycoplasma detection kit. We certify that these results meet our quality release criteria.

EC_{50} for CTP:	NA
Stability:	Cells were kept in continuous culture for 20 passages (~ 60 days) and showed no decrease in functional response in the PhotoScreen® assay (EC_{50}, E_{max}).
Mycoplasma:	This cell line tested negative for Mycoplasma.

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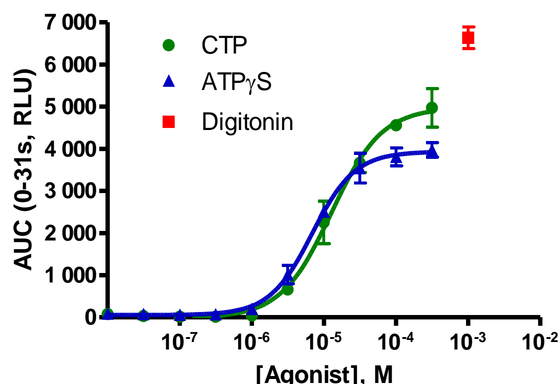
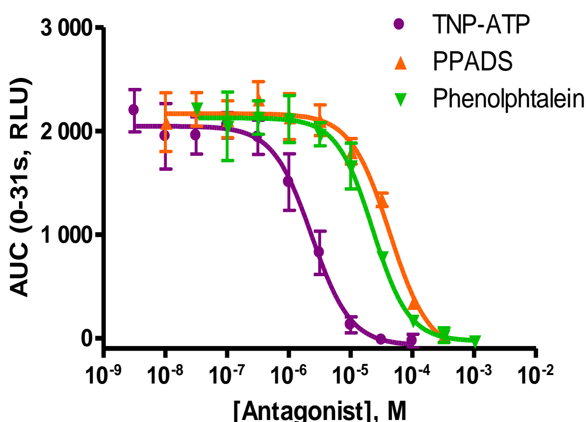
Typical Product Data – PhotoScreen® Assay



Agonist	pEC_{50}	TOP Agonist (AUC, RLU)	% of Digitonin Response
CTP	4.9	4 977	75%
ATPγS	5.2	3 932	59%

Figure 1. PhotoScreen® Agonist Dose-Responses.
Cells (20 000 cells/well) were seeded into the wells of a poly-D-Lys coated white, clear bottom, 384-well plate. Cells were loaded with 10 µM native coelenterazine in HBSS + 15 mM HEPES for 4 h at room temperature (~22°C) in the dark. Agonist dilutions, prepared in HBSS + 15 mM HEPES, were dispensed on the cells and signal was measured from seconds 0 to 31 following agonist addition using a FLIPR$^{TETRA®}$ system. Data from a representative experiment are shown. As a control, of response specificity, parental HEK-mito-Photina® cells (i.e. not transfected with the $P2X_4$ receptor) were tested in parallel, and CTP, tested up to 1 mM, did not elicit any response in these cells (data not shown). For this reason, CTP is the reference agonist that we recommend to setup screening assays with the P2RX4 cell line.



Antagonist	pIC_{50}
TNT-ATP	5.6
PPADS	4.5
Phenolphtalein	4.6

Figure 2. PhotoScreen® Antagonist Dose Responses.
Cells (20 000 cells/well) were seeded into the wells of a poly-D-Lys coated white, clear bottom, 384-well plate. Cells were loaded with 10 µM native coelenterazine in HBSS + 15 mM HEPES for 4 h at room temperature (~22°C) in the dark. Antagonists, prepared in HBSS + HEPES, were added to the cells and, after 10 min, a concentration of 90 µM of CTP (final concentration of 30 µM is the EC_{80}), prepared in HBSS + HEPES, was dispensed on the cells and signal was measured from seconds 0 to 31 following agonist addition using a FLIPR$^{TETRA®}$ system. Data from a representative experiment are shown.

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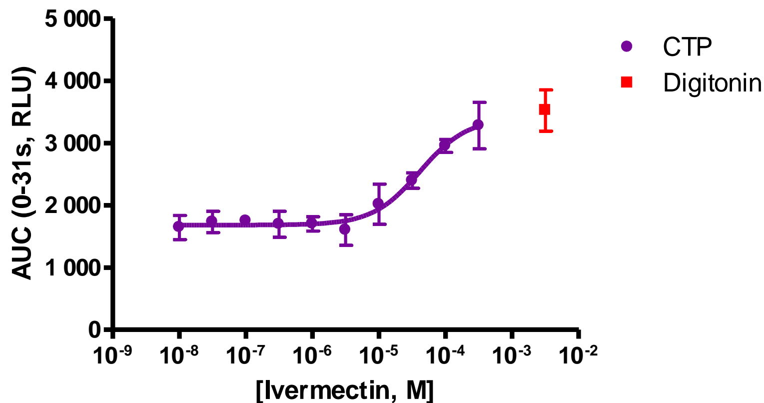

Figure 3. Potentiating effect of ivermectin on the CTP response of P2RX4 in PhotoScreen® Assay. Cells (20 000 cells/well) were seeded into the wells of a poly-D-Lys coated white, clear bottom 384-well plate. Cells were loaded with 10 µM native coelenterazine in HBSS + HEPES for 4 h at room temperature (~22°C) in the dark. Ivermectin dilutions, prepared in HBSS + HEPES, were added to the cells and, after 10 min, 20 µL of 90 µM CTP (final concentration of 30 µM) prepared in HBSS + HEPES, were dispensed on the mixture of cells + ivermectin. Signal was measured from seconds 0 to 31 following agonist addition using the FLIPR$^{TETRA®}$. Data from a representative experiment are shown.

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Typical Product Data – Electrophysiology

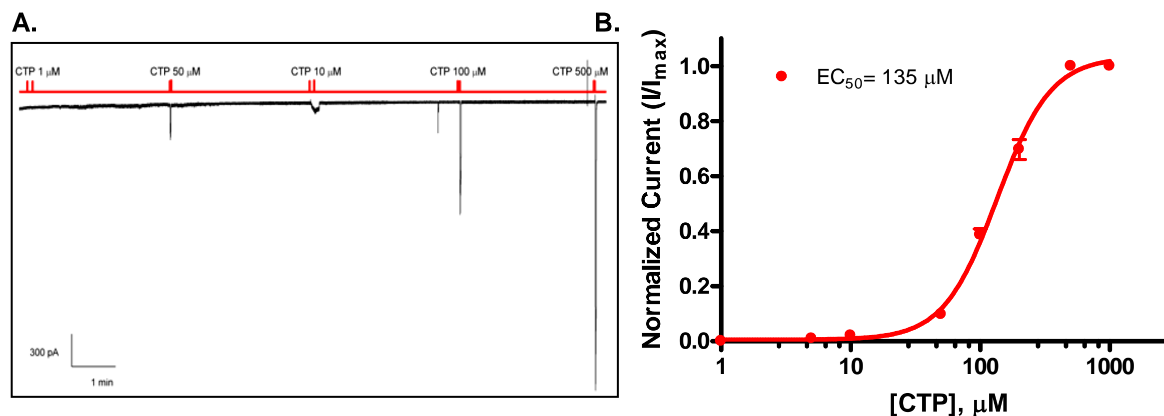

Figure 4. Agonist dose-response measured by whole cell voltage clamp.
Various concentrations of CTP were applied to cells held at a potential of -60 mV. While little desensitization was observed when 500 µM CTP was applied at 1-min intervals, full recovery was obtained within 3-min (data not shown). For this reason a 3-min interval was selected for the successive applications of various CTP concentrations as shown on panel **A** for a representative cell. The current intensity from the measurement performed on 6 cells was normalized to the one obtained for the application of 500 µM CTP to draw the dose-response illustrated in panel **B**. As a control of the specificity of the response, parental HEK cells were also tested in a whole cell voltage clamp, and application of 100 µM CTP to these cells did not elicit any current (data from 10 cells, not shown).

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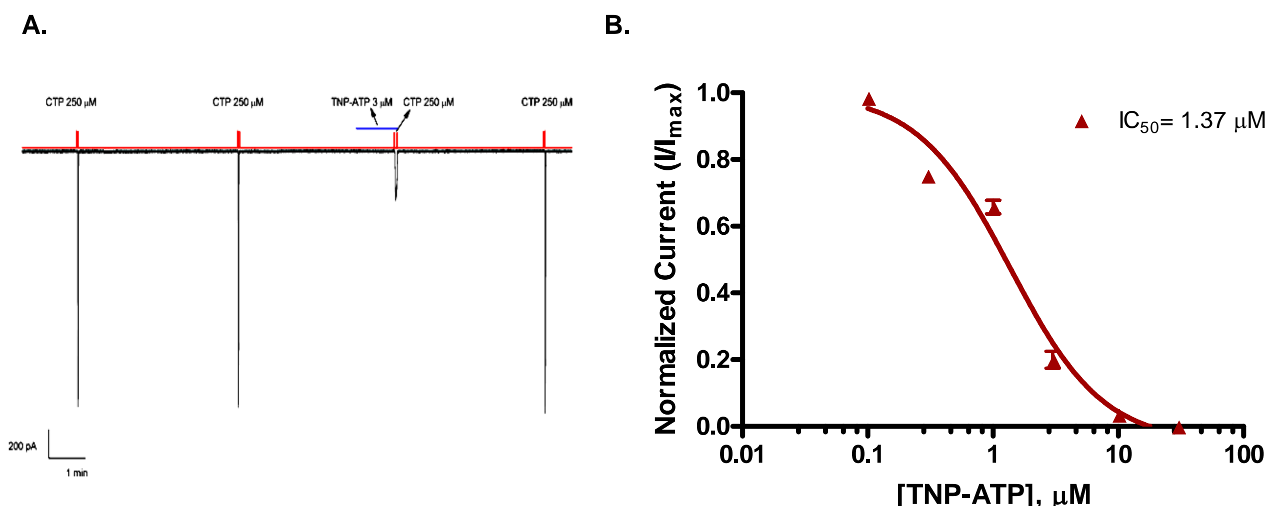

Figure 5. Antagonist dose-response measured by whole cell voltage clamp.
A concentration of 250 µM CTP (corresponding to its EC_{80} concentration) was applied to cells held at a potential of -60 mV. To study the blockage of the P2RX4 receptor by TNT-ATP, cells were perfused with the indicated concentrations of TNT-ATP for 1-min, followed by co-application of 250 µM CTP for 0.5-2 s, at 4-min intervals to allow full recovery from desensitization. Data from a representative cell are shown on panel **A**, and the current intensity from the measurement performed on 4 cells was normalized to the one obtained for the application of 250 µM CTP alone to draw the dose-response illustrated in panel **B**.

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Recommended Cell Culture Conditions

Cell Culture Medium: MEM Earles, 10% fetal bovine serum (FBS), 400 µg/mL Geneticin (receptor expression selection), 0.2 µg/mL Puromycin (Photina® expression selection), 100 IU/mL penicillin, 100 µg/mL streptomycin.

Freezing Medium: MEM Earle's, 10% FBS, 10% DMSO.

Thawing Cells: Using appropriate personal protective equipment, 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 with sterile towel. Under aseptic conditions using a pipette, transfer content to 10 mL complete medium and centrifuge (150 x *g*, 5 min). Resuspend cell pellet in 10 mL of complete medium and transfer to an appropriate culture flask (see table below). Cells are cultured as a monolayer at 37°C in a humidified atmosphere with 5% CO_2.

Cell Type	Cells/cm^2
CHO	11 000 – 15 000
HEK293	41 000 – 45 000
1321N1	19 000 – 23 000

Cell Culture Protocol: Typically, for regular cell culture maintenance, cells are grown to 80% confluence and trypsinized (0.05% trypsin / 1 mM EDTA in calcium and magnesium-free PBS).

PhotoScreen® Assay Procedure

Experimental Procedure for P2RX4 (Adhesion Mode):

1. Cells grown till mid-log phase (70-90% confluency) are detached using trypsin and seeded at a concentration of 20 000 cells/well in poly-D-Lys coated white, clear bottom assay plates in culture medium without antibiotics and with 10% FBS. Cells are let to adhere in a 37°C, 5% CO_2 incubator overnight. **Note: When working with the LumiLux®, black, clear bottom assay plates are used instead of white, clear bottom plates.**
2. Medium is removed by plate overthrow and tapping on a paper towel, then 20µL/well of HBSS +15 mM HEPES containing 10 µM (FLIPR$^{TETRA®}$) or 5 µM (Lumilux Cellular Screening platform) native coelenterazine is added to the cells and plates are incubated for 4 h at room temperature in the dark.
3. For the agonist assay, using the reader's automatic injection system, dispense in triplicate on the coelenterazine-loaded cells 20 µL/well of agonist at the desired concentrations, diluted in HBSS + 15 mM HEPES, and record the relative light emission for the desired time interval. Digitonin at a final concentration of 100 µM diluted in assay medium is used to measure the receptor independent cellular calcium response.

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4. For the antagonist assay, dispense in triplicate on the coelenterazine-loaded cells 20 µL/well of antagonist at the desired concentrations, diluted in HBSS + 15 mM HEPES. After 10 min of incubation, using the reader's automatic injection system, inject 20 µL of the reference agonist at a final concentration equivalent to the EC_{80}, prepared in HBSS + 15 mM HEPES, and record the relative light emission for the desired time interval.

Note #1: *This assay was also validated on the LumiLux® system in adherent mode.*

Note #2: *Using a suspension cells protocol with this cell line is not possible, as the release of free nucleotides by the cell suspension is expected to desensitize the P2RX4 ion channel.*

Electrophysiology – Whole Cell Voltage Clamp

Intracellular solution: 130 mM L-aspartic acid potassium salt, 10 mM NaCl, 2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 10 mM EGTA (final Ca^{2+} free concentration is 14 nM) and 10 mM HEPES, pH 7.3

Extracellular solution: 130 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES and 5 mM D-Glucose, pH 7.3

Protocol:
For all the experiments, 50 000 cells were plated in 35-mm plastic culture dishes the day before the experiment. Cells were held at -60 mV and currents were elicited by perfusion of an agonist of P2RX4 through a 9-hole (0.6 mm) rotating perfusion system (Biologic, Grenoble, France) placed near the cell under study (average response time of 0.5 -1 second). The flow rate was constant at 120 µL/min. The whole cell clamp success rate was 100% (n=73).

Data Acquisition:
Standard whole-cell voltage clamp experiments were performed at room temperature. For data acquisition and further analysis we used the MultiClamp 700A (Axon Instruments, USA). pClamp 8.2 (Axon Instruments) and Origin 7 (Microcal Inc.) softwares were routinely used during data acquisition and analysis. The data were filtered at 400 Hz (-3 dB Bessel low-pass). Sampling interval per signal was 1 ms.

- Liquid junction potential: No correction.
- Series resistance: Cell capacitance and series resistance errors were carefully compensated for (85–90%) in order to reduce the voltage errors to <5% of the protocol pulse.
- Pipette resistance and cell capacitance: pipettes' resistance was 1.7-2.4 MΩ. 24 hours after seeding, the mean capacitance of the cells was 18.97 ± 1.38 pF (n=20).
- Current density: The mean current density (at -60 mV and 500 µM CTP) was 110.42 ± 11.62 pA/pF (n=21).

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References

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Materials

The following tables give the references of compounds and reagents used for the characterization of the P2RX4 cell line, as well as some advice on how to use these compounds:

Table 1. Recommended compounds for functional characterization

Name	Provider	Cat n°	Stock Solution	Remarks
CTP	SIGMA	C1506	100 mM in water	P2RX4 Agonist
ATPγS	SIGMA	A1388	45.7 mM in water	P2RX4 Agonist
TNP-ATP	Tocris	2464	10 mM in water	P2RX4 Antagonist
PPADS	Tocris	0625	100 mM in water	P2RX4 Antagonist
Phenolphthalein	SIGMA	P9750	50 mM in DMSO	P2RX4 Antagonist
Ivermectin	Tocris	1260	100 mM in DMSO	P2RX4 Positive Modulator
Digitonin	FLUKA	37006	50 mM in DMSO	Detergent used for determination of maximal PhotoProtein signal
Native coelenterazine	Promega	S2001	1 mM in methanol	Use for reconstitution of active Photina®
Carbachol	SIGMA	C2409	100 mM in water	Agonist of endogenous muscarinic receptor of HEK cells, can be used as a positive control in the photoprotein assay

Table 2. Cell culture media and assay buffers

Name	Provider	Cat n°	Remarks
MEM Earles with glutamine	Hyclone	SH30024.02	For cell culture and freezing
FBS	Wisent	080150	For cell culture and freezing
Puromycin	Wisent	400-160	For cell culture
Geneticin/G-418	Wisent	400-130	For cell culture
Standard HBSS (with $CaCl_2$ and $MgCl_2$)	Invitrogen	14025	For photoprotein assay
HEPES	Invitrogen	15630	For photoprotein assay

For other materials and instrumentation, please visit our website

www.perkinelmer.com/GPCR

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