B'SYS GmbH

HEK TRPM8 Cell Line

Specification Sheet

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1 BACKGROUND

1.1 The TRPM8 channel

The transient receptor potential melastatin TRPM8 vanilloid receptor gates a Ca^{2+} permeable cation channel that is over expressed in prostate cancer and other malignancies. TRPM8 channels can be activated by cold and cooling compounds such as menthol and icilin (Bödding et al., 2007).

1.2 B'SYS's HEK TRPM8 Cells

B'SYS has designed a new HEK TRPM8 cell line with constitutive expression of human TRPM8 channel. The human TRPM8 cDNA was cloned and transfected into HEK 293 cells and then the functional properties of the TRPM8 channel was validated by means manual and automated (Qpatch™) patch-clamp recordings. The validation was made using menthol. The TRPM8 antagonists SKF96365 or PF-05105679 were also used to block the channel at a single concentration. Results are outlined in section 3.

2 PRODUCT SHIPMENT

2.1 Product Format

HEK 293 cells stably transfected with recombinant human TRPM8 channel:

- 1x 0.5 mL aliquots of frozen cells at 2.4 E+06 cells/mL
- Cells are frozen in complete medium with 10% DMSO

2.2 Mycoplasma Certificate

B'SYS periodically tests cells for presence of mycoplasma by means of highly sensitive PCR based assays. All delivered cells are free of mycoplasma.

3 VALIDATION OF HEK TRPM8 CELLS

3.1 Electrophysiology

TRPM8 currents were measured by means of the patch-clamp technique in the whole-cell configuration. Manual patch-clamp: The bath solution contained (in mM) NaCl 137, KCl 4, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 10, D-Glucose 10. The pH was adjusted to 7.4 with NaOH. The pipette solution consisted of (in mM) CsCl 145, NaCl 8, $MgCl_2$ 1, Mg-ATP 2, EGTA 5, HEPES 10. The pH was adjusted to 7.20 with CsOH. After formation of a GΩ seal between the patch electrodes and individual TRPM8 stably transfected HEK cells, the cell membrane across the pipette tip was ruptured to assure electrical access to the cell interior. As soon as a stable seal could be established the cells were clamped at -60 mV and the TRPM8 currents were activated by the application of different concentrations of menthol. TRPM8 currents elicited by 100 µM menthol were blocked by the agonist SKF96365 (1 µM) to control cell sensitivity.

Automated patch-clamp: The bath solution contained (in mM) NaCl 137, KCl 4, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 10, D-Glucose 10. The pH was adjusted to 7.4 with NaOH. The intracellular solution consisted of (in mM) KCl 130, $CaCl_2$ 2, $MgCl_2$ 4, Na_2ATP 4, EGTA 5, HEPES 10. The pH was adjusted to 7.20 with KOH. TRPM8 currents were measured upon application of menthol (30 µM) to patch-clamped cells. The cells were voltage-clamped at a holding potential of -60 mV and every 5 s a voltage ramp from -60 mV to 60 mV (duration of 1000 ms) was applied:

3.2 Patch-clamp success rates

The patch-clamp properties of the HEK TRPM8 cell line were elucidated at typical working passage numbers (passage 22-26). A total of 16 cells were analyzed. Success for establishment of on-cell configuration was defined as follows: > 1 GΩ. The whole-cell configuration was not accepted if the membrane resistance was below 500 MΩ. A successful recording had to be free of rundown effects and variations in series resistance.

- On-cell successful: **94%** (n=15)
- Whole-cell successful: **94%** (n=15)
- Recording (15 min) successful: **75%** (n=12)

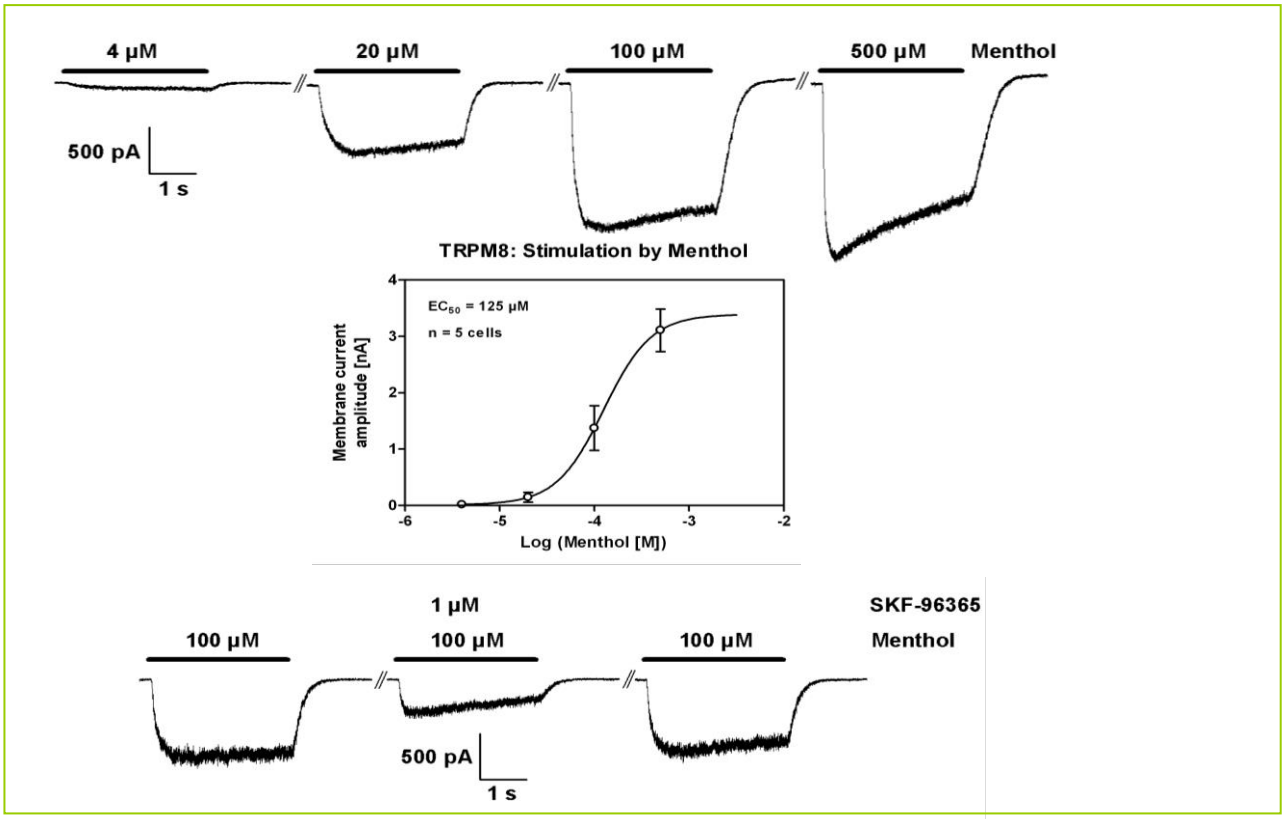

Fig.1: From top to bottom: Activation of TRPM8 currents by increasing concentrations of menthol (4 µM to 500 µM) / Dose response curve of menthol on TRPM8 current fitted to data from n=5 cells / Block of menthol-activated TRPM8 channels by 1 µM SKF-96365.

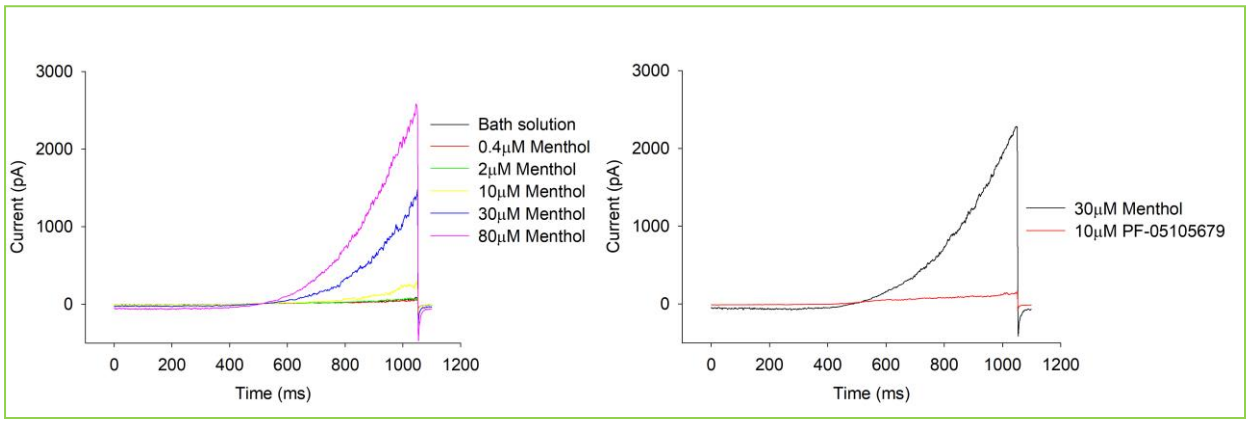

Fig.2: Automated patch clamp recordings. Left graph: Representative current traces showing increasing, cumulative applications of the agonist menthol. Right: Representative current traces showing inhibition of TRPM8-mediated currents by the antagonist PF-05105679.

4 CELL CULTURE CONDITIONS

4.1 General

HEK TRPM8 cells are incubated at 37°C in a humidified atmosphere with 5% CO_2 (rel. humidity > 95%). The cells are continuously maintained and passaged in sterile culture flasks containing DMEM medium supplemented with 9% fetal bovine serum, 0.9% Penicillin/Streptomycin solution and 1 µg/mL Puromycin HEK TRPM8 cells are passaged at a confluence of about 80%. For electrophysiological measurements the cells are seeded onto e.g. 35 mm sterile culture dishes containing complete medium.

- All solutions and equipment coming in contact with the cells must be sterile.
- Use proper sterile technique and work in a laminar flow hood.
- Be sure to have frozen cell stocks at hand before starting experiments.
- Cells should be split every 2-3 days at 70% - 80% confluency at 1:3 to 1:5 ratio.

Table 1: Cell culture reagents

Product	Supplier	Order number
DMEM/Nutrient mixture F-12 Ham (DMEM/F12)	Sigma-Aldrich	D8437
Fetal Bovine Serine (FBS)	Gibco	10270-106
Penicillin / Streptomycin (100x)	Gibco	10378-016
Phosphate Buffered Saline (PBS, without Ca^{2+} and Mg^{2+})	Sigma-Aldrich	D8537
Puromycin (10 mg/mL)	Gibco	A1113803
Detachin	Genlantis	T100T100
Trypsin EDTA (10x)	Sigma-Aldrich	T4174
DMSO	Sigma-Aldrich	D2438

For the preparation of 1X Trypsin/EDTA, the 10X solution is diluted in PBS (without Ca^{2+} and Mg^{2+}), aliquoted and stored in the freezer.

4.2 Recommended Complete Medium

- 500 mL DMEM/F12 with L-Glutamine or GlutaMax I
- 10% FBS
- 1.0% Penicillin/Streptomycin

4.3 Antibiotics

- HEK TRPM8 clones were selected under 1 µg/mL Puromycin antibiotic pressure.
- To cultivate HEK TRPM8 cells, the same antibiotic pressure (1 µg/mL) should be used.
- To separate HEK TRPM8 cells from untransfected cells, use 1 µg/mL Puromycin

Remark: The permanent application of high antibiotic pressure has no effect on current density.

4.4 Thawing Cells

- Remove vial of cells from liquid nitrogen and thaw quickly at 37°C.
- Decontaminate outside of vial with 70% Ethanol.
- Transfer cells to a T-25 culture flask containing 5 mL complete medium
- Incubate cells at 37°C for at least 4-6 hours to allow the cells to attach to the bottom of the flask
- Once cells attach to the bottom of the flask and look healthy, aspirate off the medium and replace with 5 mL complete medium containing selection antibiotics for cultivation (see **Error! Reference source not found.**)
- To check whether cells are attached properly, the flask can be gently moved while looking under the microscope
- If 48h after thawing the confluency is below 50%, replace the medium in the flask with fresh medium containing antibiotics
- Incubate cells at 37°C and check them daily until 50% to 80% confluency is reached.

4.5 Splitting Cells

- When cells are 50% to 80% confluent remove complete medium.
- Wash cells with 1xPBS to remove excess medium
- Add 0.5 mL to 1 mL Detachin (or 1x Trypsin/EDTA) and incubate for 2 min at 37°C.
- Detach cells by gently tapping the sides of the flask add complete medium and pipet up and down to break clumps of cells.
- Passage cells into new flask with complete medium and antibiotics at 1:3 to 1:5 ratio.
- Use remaining suspension for counting the cells.

4.6 Freezing Medium

- Mix 0.9 mL fresh complete medium and 0.1 mL DMSO for every 1 mL freezing medium.
- Sterilize freezing medium by means of appropriate micro filter (0.1 µm – 0.2 µm).

4.7 Freezing Cells

- Prepare fresh freezing medium and keep it on ice.
- Cells should have 80% - 90% confluency prior to freezing.
- Remove the complete medium and wash cells with 1xPBS.
- Add 0.5 mL to 1 mL Detachin (or 1x Trypsin/EDTA) and incubate for 2 min at 37°C
- Detach cells by gently tapping the sides of the flask, add complete medium and pipet up and down to break clumps of cells.
- Pellet cells at 200 g using a centrifuge and carefully aspirate off medium.
- Resuspend cells at a density of approximately 1.0 E+06 cells per mL with fresh freezing medium.
- Aliquot 0.75 mL of cell suspension into each cryovial.
- Overnight incubate cells in a polystyrene box at –80°C.
- The next morning transfer cryovial in liquid nitrogen tank for long-term storage.

4.8 Stability of HEK TRPM8 cells

HEK TRPM8 cells stably express functionally active TRPM8 channels over 35 passages. Under recommended cell culture conditions no variation in current density was observed over 35 cell splitting cycles.

5 TRPM8 SEQUENCE

5.1 Human TRPM8 Accession Number NM_080704.2

Cloned cDNA sequence of human TRPM8 subunit was error-free and identical with NM_024080.3 sequence:

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