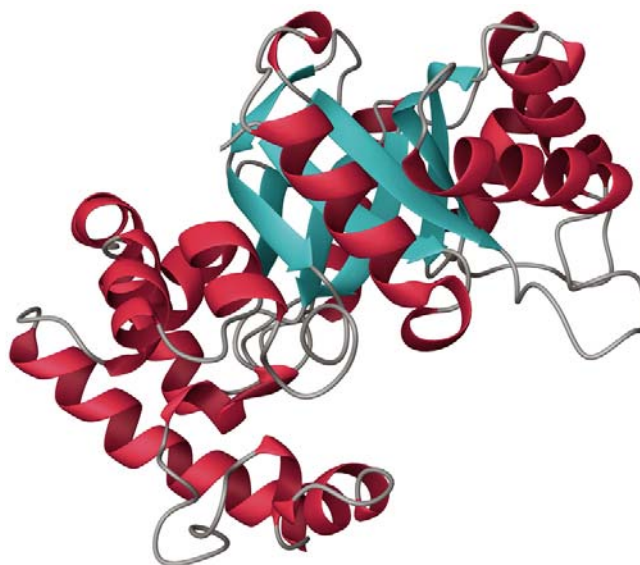


hTRPM8-HEK293
Recombinant Cell Line

cat. #CYL3048

Revision 1



Ordering Information and Technical Services:

MILLIPORE (UK) LIMITED
6-7 Technopark
Cambridge
CB5 8PB
UK

Tel: +44 (0) 1223 508191
Fax: +44 (0) 1223 508198

Customer Services UK: 0800 0190 333
US: 800 437 7500

www.millipore.com/ionchannels

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Licensing Statement

The CMV promoter is covered under U.S. Patents 5,168,062 and 5,385,839 and its use is permitted for research purposes only. Any other use of the CMV promoter requires a license from the University of Iowa Research Foundation, 214 Technology Innovation Center, Iowa City, IA 52242, USA.

Use of IRES is covered by U.S. Patent 4,937,190 and is limited to use solely for research purposes. Any other use of IRES requires a license from Wisconsin Alumni Research Fund (WARF).

The bovine growth hormone (bgh) polyadenylation signal is patented under U.S. Patent No. 5,122,458. Use, in the USA, of the bgh polyadenylation signal found in screening systems sold by Millipore requires a license from Research Corporation Technologies, Inc. (RCT). After purchasing these materials from Millipore, you must contact RCT within 30 days to obtain a commercial license. The bgh polyadenylation signal cannot be used until a commercial license is obtained. Contact Jennifer Caldwell, Ph.D., at Research Corporation Technologies, Inc., 101 North Wilmot Road, Suite 600, Tucson, AZ 85711-3335, USA. Tel: 1-520-748-4400, Fax: 1-520-748-0025.

Product description:

Recombinant HEK293 cell line expressing the human transient receptor potential cation channel protein (TRPM8).

Format:

2 x 1 ml aliquots containing 1.70×10^6 cells/ml in 10% DMSO at passage 7.

Mycoplasma Testing:

The cell line has been screened using the ELISA based Mycoplasma Detection kit (Roche) and by a PCR VenorGem kit (Minerva Biolabs) to confirm the absence of Mycoplasma species.

Functional Validation:

Manual patch recording of the cell line showed the currents to be tonically active at room temperature. At voltages positive to 0 mV the current deviates from linearity rectifying in an outward direction.

This tonically-active current was completely and reversibly blocked by 1 μM BCTC (*N*- (4-tert. butyl-phenyl) - 4 - (3-chloropyridin-2-yl) tetrahydropyrazine - 1 (2*H*)-carboxamide).

Bath application of icilin evoked currents that were rapidly and irreversibly desensitized.

Using a voltage ramp protocol on IonWorks™ HT we obtained an estimation of the icilin-evoked current. There was marked outward rectification, with little inward current negative to the reversal potential of 0 mV.

Increases in $[\text{Ca}^{2+}]_i$ were recorded on the FlexStation. Icilin increased $[\text{Ca}^{2+}]_i$ with an EC_{50} of 0.096 μM . BCTC blocked a 0.3 μM icilin-evoked response with an IC_{50} of 1.45 μM . Both these values are in line with those reported in the literature.

The $[\text{Ca}^{2+}]_i$ signal as measured on the FlexStation was stable for > 30 passages with a signal of > 300% above the baseline.

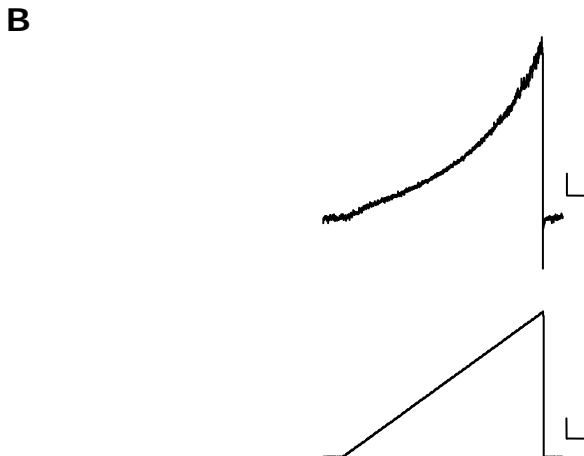
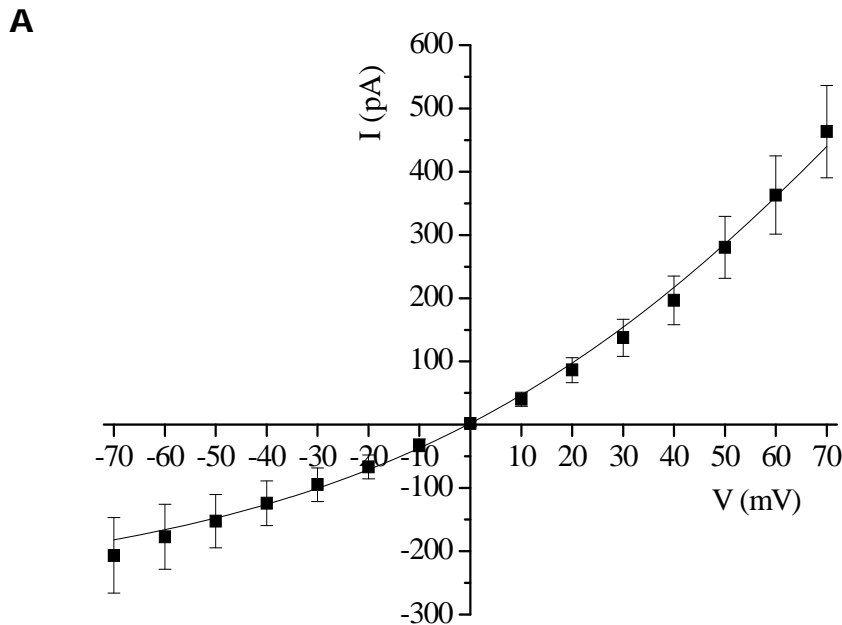
Electrophysiological Properties of the hTRPM8 Current.**Conventional Whole-Cell Patch Clamp Electrophysiology and IonWorks™ HT.****Current/Voltage Relationship:**

Membrane currents typical of TRPM8 were recorded, by whole-cell patch clamp electrophysiology, at room temperature in the absence of its agonist icilin due to the fact that the current is tonically active at temperatures below 26°C as previously reported (Chuang *et al.*, 2004). Initial characterization was of this basal current to ensure it had the biophysical properties of TRPM8.

Figure 1. Current-voltage (I/V) relationship of the tonically activated current.

A. I/V plot of the tonically active current (mean = 7 cells). At voltages positive to 0 mV the current deviates from linearity, rectifying in an outward direction.

B. A typical current (upper panel) evoked by voltage-ramp protocol from -70 to +70 mV (lower panel) illustrating outward rectification in response to a depolarizing voltage ramp. Note that during the ramp the membrane current becomes increasingly 'noisier' as the cell is depolarized. Scale bars represent 100 ms and 50 pA (x-axis and y-axis respectively - upper panel) and 100 ms and 20 mV (x-axis and y-axis respectively - lower panel).

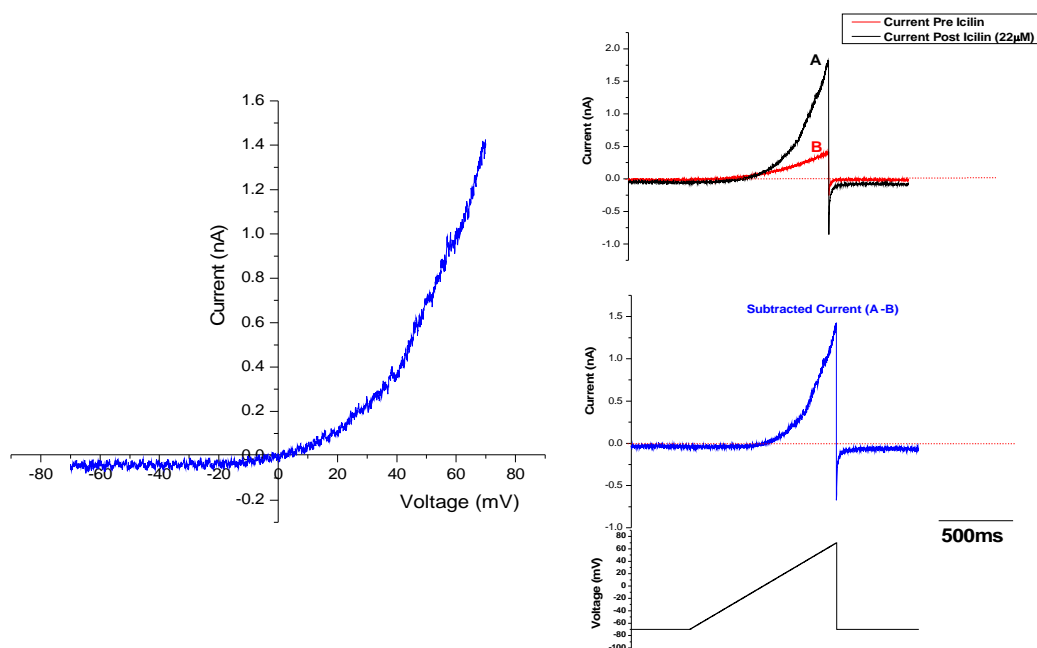


These data are similar to the observations described in **Figure 2** for icilin-evoked current on IonWorks™ HT. Therefore it is likely that the tonically active recorded currents with manual patch also represent TRPM8. Utilising a voltage ramp during the IonWorks™ HT experimental protocol also enabled us to obtain an estimation of the current-voltage (I/V) relationship of the icilin-evoked current.

Figure 2. A voltage ramp was used to obtain approximate I/V relationship.

Right-hand panel: membrane currents evoked by the voltage ramp (bottom) before and on addition of 22 μ M icilin (top; red and black traces respectively). Subtracting these traces obtains the icilin-evoked current (middle; blue trace).

Left-hand panel: icilin-evoked current plotted against voltage. The I/V relationship shows marked outward rectification with very little inward current flowing negative to the reversal potential of approximately 0 mV. These features are typical of TRPM8 currents described in the literature.

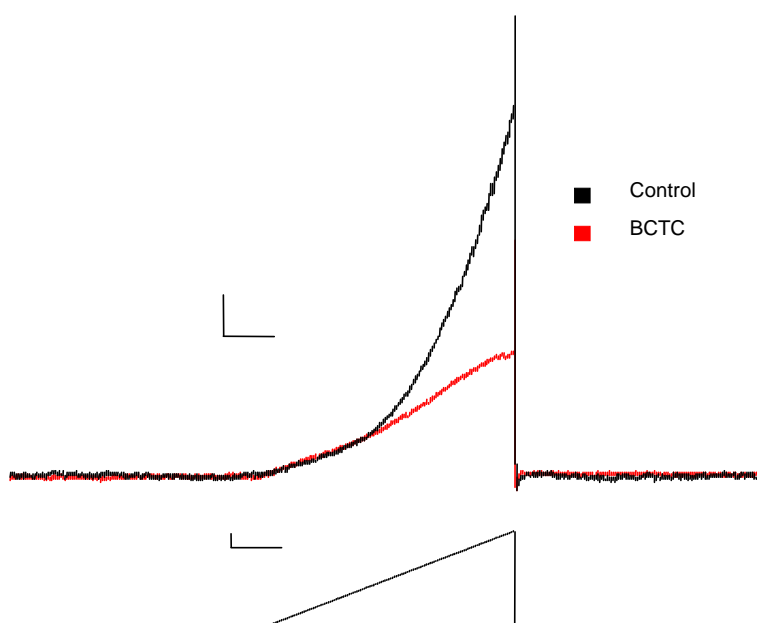


Pharmacological properties of the hTRPM8 Current.

Conventional Whole-Cell Patch Clamp Electrophysiology.

Further evidence that the tonically-activated current is indeed TRPM8 is provided in **Figure 3**. We examined the effect of BCTC (*N*-(4-tert.butyl-phenyl)-4-(3-chloropyridin-2-yl) tetrahydropyrazine-1(2*H*)-carboxamide) (0.1-1 μ M) a potent VR1 (TRPV1) antagonist known to block TRPM8 activation (Behrendt *et al.*, 2004; Weil *et al.*, 2005). BCTC completely blocked the outwardly rectifying component of the tonically-activated current, leaving an essentially linear I/V that reflects the linear non-specific leak of the cell. (i.e. BCTC at this concentration completely blocked the tonically-activated current).

Figure 3. Inhibition of tonically-activated hTRPM8 currents by BCTC (1 μ M). Whole-cell current traces of hTRPM8 activated at room temperature (upper black trace) evoked by 1 s voltage-ramp from -70 to +70 mV and in the presence of 1 μ M BCTC (red trace). Upper panel scale bars represent 200 ms (x-axis) and 100 pA (y-axis). Lower panel scale bars represent 200 ms (x-axis) and 20 mV (y-axis).

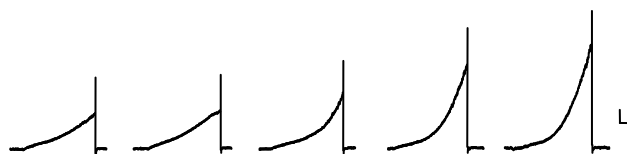
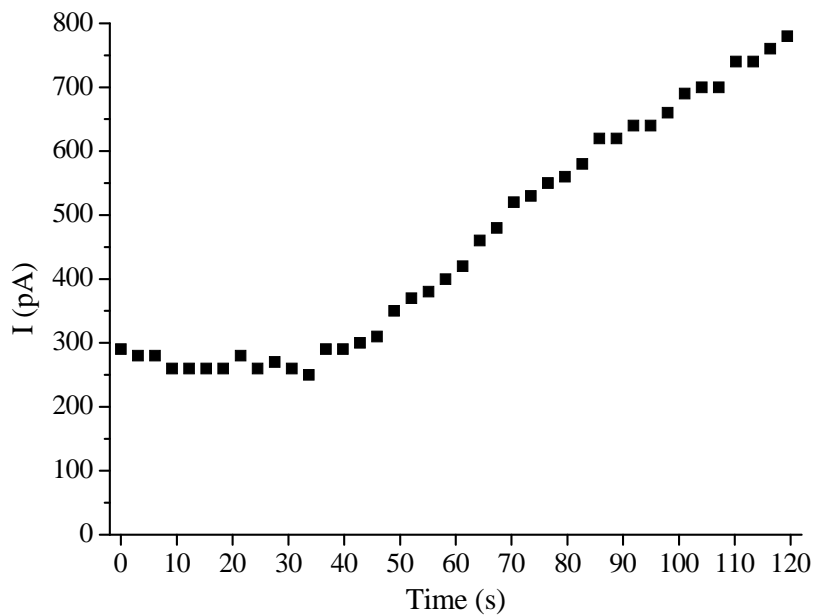


These BCTC effects were shown to be fully reversible (see **Figure 4**). The upper graph shows current values measured at +70 mV at various time points after removal of BCTC. Initially, (0 - 30 s) after removal of BCTC, the current at +70 mV is around 300 pA and primarily represents leak current. At time points >40 s the outward current becomes progressively larger, until after 120 s the outward current at +70 mV is around 800 pA.

Figure 4. Recovery of TRPM8 currents from the inhibitory effect of BCTC.

Upper panel: Currents were measured at +70 mV (evoked by a voltage-ramp protocol from -70 to 70 mV).

Lower panel: Current traces 0, 30, 60, 90 and 120 seconds after removal of 1 μ M BCTC. Scale bars represent 200 ms (x-axis) and 100 pA (y-axis).

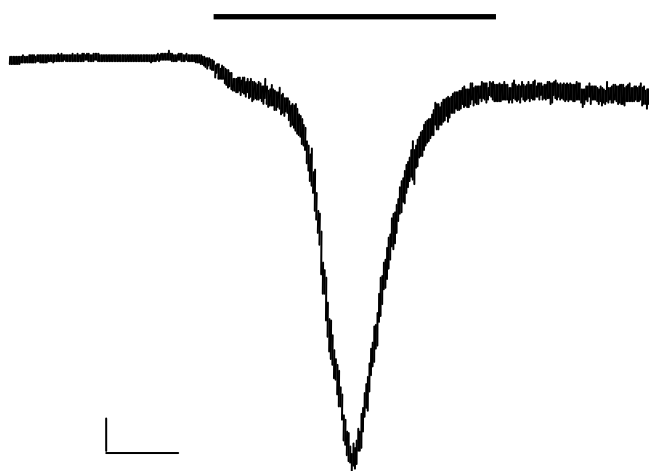


Icilin-activated current:

Initially, the intention was to evoke TRPM8 using icilin and obtain an EC_{50} value. However, because there was significant tonic activation of the current (see above) and the current irreversibly desensitized to bath application of icilin, this proved technically unfeasible given the time constraints. **Figure 5** illustrates the desensitizing effect of 30 μ M icilin. Subsequent additions of icilin to this cell failed to evoke any further responses.

These findings are typical when using icilin as reported by Andersson *et al.*, 2004; and Chuang *et al.*, 2004 who found that icilin activates TRPM8 with a short lag (~ 10 s) and desensitises rapidly and completely.

Figure 5. Icilin activated evoked currents in TRPM8. Bath application of icilin (30 μ M) was added at the horizontal bar. The current was recorded at a holding potential of 40 mV. Scale bars represent 10 seconds (x-axis) and 100 pA (y-axis).



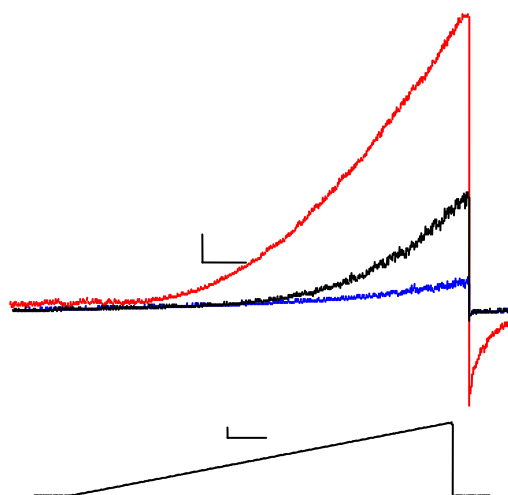
In order to characterize the icilin evoked response, voltage ramps were applied from -70 mV to +70 mV (see above), before (Figure 6, black trace), at the peak (red trace) and after desensitization, during the continued presence of icilin (blue trace). What is clearly evident is that the tonically activated current is similarly desensitized by icilin so that after the desensitization process has taken place the ramp current (blue trace) is much smaller than the current trace *prior* to icilin addition. This supports the notion that the icilin evoked and tonically activated channel are functionally identical as expected.

Figure 6. Whole-cell current-voltage relationship of TRPM8 activated by icilin.

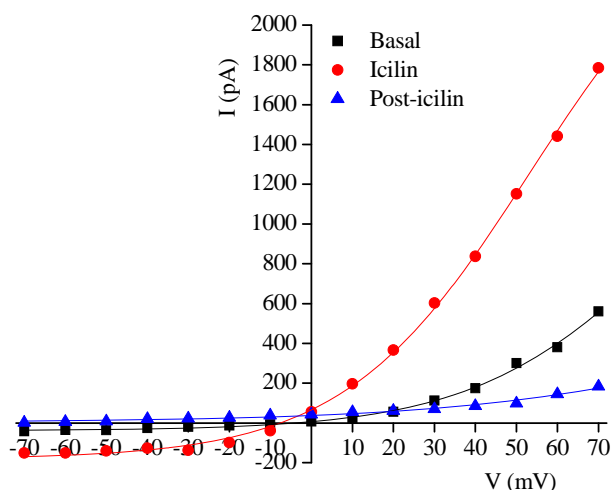
A. Whole-cell current traces (upper traces) evoked by 1 s voltage-ramp protocol from -70 to +70 mV in the absence (black), presence of 30 μ M icilin (red) and after wash-off of icilin (blue). Scale bars represent 100 ms and 200 pA (x-axis and y-axis respectively - upper panel) and 100 ms and 20 mV (x-axis and y-axis respectively - lower panel).

B. Whole-cell current-voltage relationship of the hTRPM8 from ramp traces.

A



B



Pharmacological properties of the hTRPM8 Current.

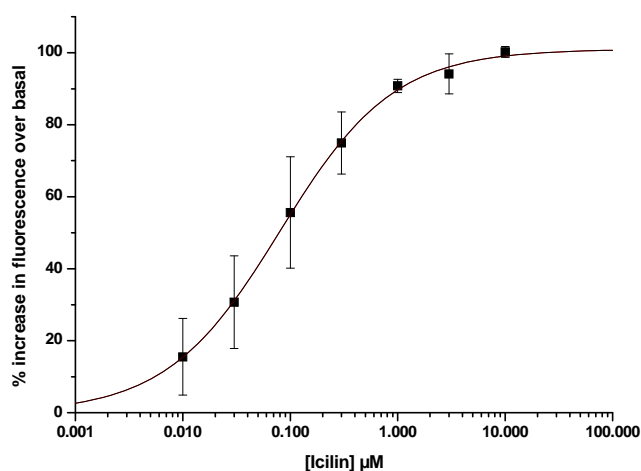
FlexStation.

The effect of the agonist icilin and the antagonist BCTC on release of intracellular calcium was determined by the measurement of Fluo4 fluorescence on the FlexStation. Experiments were conducted at 37°C to minimise the activation of the channel at lower temperatures (<26°C).

Icilin-evoked increases in $[Ca^{2+}]_i$:

Icilin was seen to increase levels of $[Ca^{2+}]_i$ in a dose-dependent manner (**Figure 7**). The EC_{50} value for icilin was 0.096 μM (n=5).

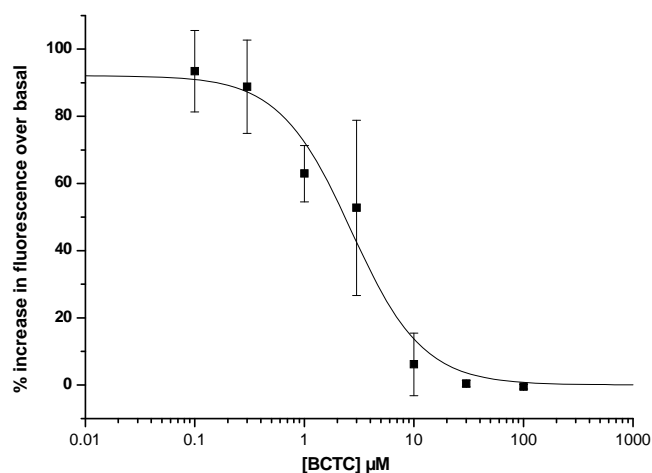
Figure 7.



Block of icilin response by BCTC:

The 0.3 μM icilin-evoked increases in $[Ca^{2+}]_i$ were blocked by BCTC in a dose-dependent manner (**Figure 8**). The IC_{50} value for BCTC was 1.45 μM (n=3-4).

Figure 8.



The EC₅₀ and IC₅₀ values of icilin and BCTC respectively are listed in **Table 1**. The EC₅₀ value of icilin is in very close agreement with those previously reported in the literature (Behrendt *et al.*, 2004 and Andersson *et al.*, 2004) whilst the IC₅₀ value for BCTC is higher (Andersson *et al.*, 2004 and Weil *et al.*, 2005).

Table 1. EC₅₀ and IC₅₀ values of icilin and BCTC.

	EC ₅₀ Icilin (μM)	IC ₅₀ BCTC (μM)
TRPM8-HEK Clone	0.096	1.45
Literature	0.2 * 0.125 ★	0.8 * 0.143 ◆

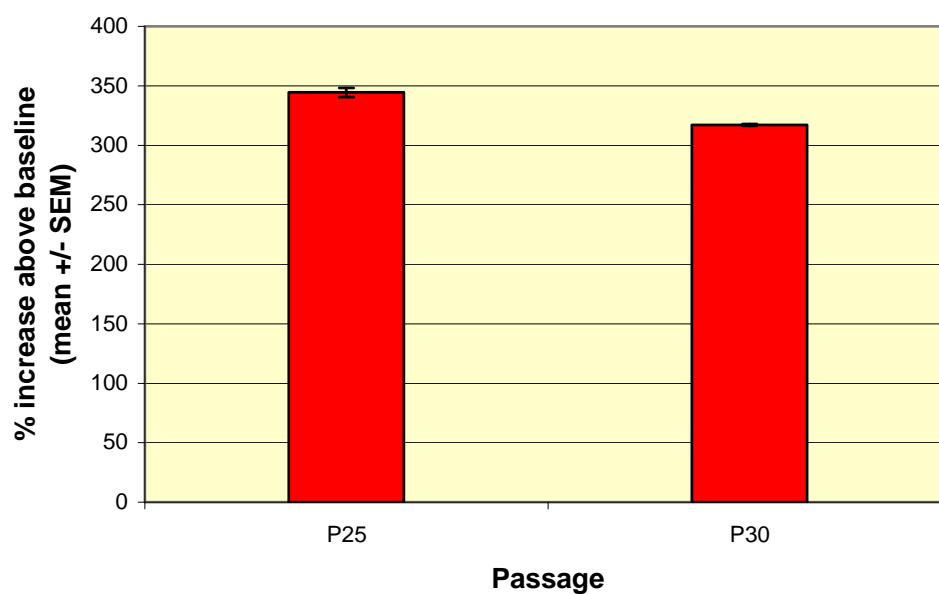
* Behrendt *et al.*, 2004.

★ Andersson *et al.*, 2004.

◆ Weil *et al.*, 2005.

Stability of hTRPM8-HEK293 Cell Line:

Figure 7. Stability of signal at passages 25 and 30. The increase in $[Ca^{2+}]_i$ evoked by application of 25 μ M icilin as measured by Fluo4 fluorescence on the FlexStation expressed relative to the baseline signal.



Recommended Culture Conditions:

Cells should be grown in a humidified environment at 37°C under 5% CO₂ using DMEM/F12 medium supplemented, 10% Foetal Bovine Serum, 1% Non Essential amino acids, plus 400 µg/ml of Geneticin to ensure that the recombinant expression is maintained.

Transfection of HEK293 host cells with the human TRPM8 construct does not appear to have retarded the growth characteristic of the host cells, which exhibit a normal cell division time of approximately 24 hours.

It is recommended to rapidly thaw a frozen aliquot from liquid nitrogen by agitation in a 37°C water-bath and to transfer vial contents into a T175 cm² flask containing 50 ml of pre-equilibrated media according to the formulation below. Allow cells to adhere for 4-8 hours at 37°C under 5% CO₂ before gently removing the media and replacing with 30 ml of fresh media.

The cell line should not be allowed to exceed 80% confluence within the culture vessel, to prevent contact inhibition causing senescence. Passage every 2-3 days by rinsing with PBS before harvesting with Trypsin/EDTA and seeding into new flasks at a density of 1-1.5×10⁶ cells per T75 cm² or 2-3×10⁶ cells per T175 cm² flask. It is essential that the cell line is continually maintained in the presence of Geneticin (400 µg/ml) which should be added to the culture vessel or media immediately prior to use.

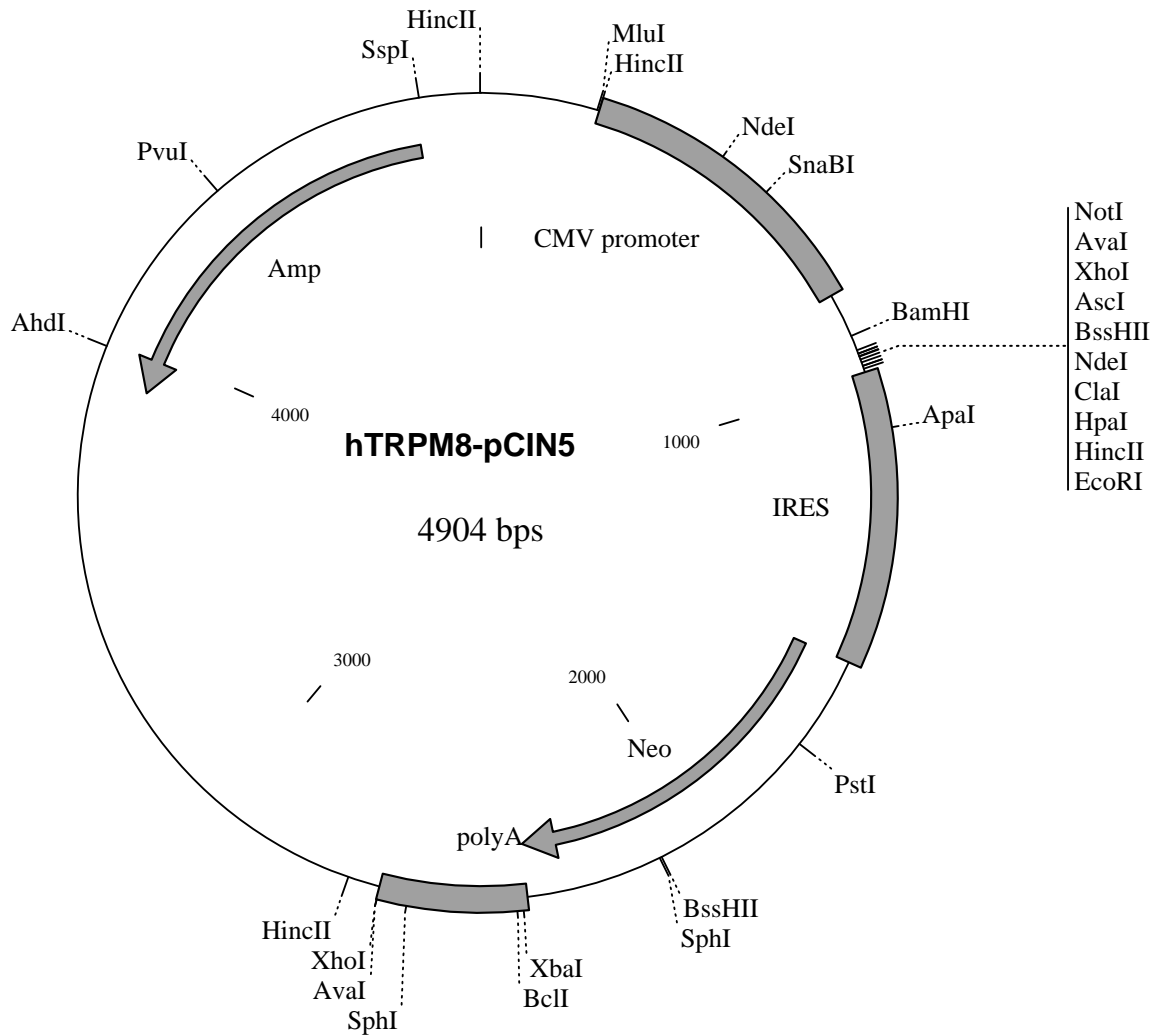
Media Formulation:

D-MEM/F-12 (with L-Glutamine)	(Invitrogen	#11320)
10% USA Certified Foetal Bovine Serum	(Invitrogen	#16000)
1% Non-Essential amino acids (100X)	(Invitrogen	#11140)
400 µg/ml Geneticin	(Invitrogen	#10131)

Other reagents required:

Trypsin/EDTA	(Invitrogen	#25300)
PBS	(Invitrogen	#14190)
Trypan Blue	(Sigma	#T8154)
DMSO	(Sigma	#D2650)

Vector:



Polylinker: CMV-BamHI-NotI-**TRPM8**-AscI-ClaI-HpaI-EcoRI-IRES-*neo*

hTRPM8 Sequence (Accession Number AY090109):

The sequence of the cDNA used to make this cell line contains one silent mutation with respect to the accession number - TGI-TGC (Cys) - at position 1968.

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TAA
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