

B'SYS GmbH

HEK-293 Ca_v1.2 Cells

Specification Sheet

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1 BACKGROUND

1.1 The cardiac L-type I_{Ca} current is encoded by Ca_v1.2

Around 20 different ionic currents account for the cardiac action potential. Briefly, these can be divided into depolarizing and repolarizing currents. The plateau phase of the cardiac action potential is mainly maintained by inward calcium flux through voltage-gated L-Type Ca_v1.2 channels. Mutations in the CACNA1C gene can cause the Long QT Syndrome with Syndactyly: Timothy Syndrome.

1.2 B'SYS' HEK-293 Ca_v1.2 Assay

B'SYS has designed a HEK-293 Ca_v1.2 cell line with stable expression of human Ca_v1.2 calcium channels. The human Ca_v1.2 α , β 2 and α 2 δ 1 subunits cDNA were cloned and cotransfected into HEK-293 cells and then the functional properties of the Ca_v1.2 channels validated by means of the patch-clamp technique. Results are outlined in section 2.

2 VALIDATION OF HEK-293 Ca_v1.2 CELLS

2.1 Biophysical Validation

Ca_v1.2 currents were measured by means of the patch-clamp technique in the whole-cell configuration. The bath solution contained (in mM) NaCl 100, KCl 4, NMDG 40, CaCl₂ 5, MgCl₂ 1, HEPES 10, Glucose 5 and Sorbitol 5. The pH was adjusted to 7.40 with HCl. The pipette solution consisted of (in mM) CsCl 93.75, Cs Methansulfonate 27, NaCl 7.5, CaCl₂ 0.55, TEA-Cl 5, MgCl₂ 6.6, Phosphocreatine Na₂ 1.2, Creatine 1.13, Pyruvate 1.13, Oxalacetate 1.13, Na₂ATP 1, HEPES 13.5, EGTA 9.25. The pH was adjusted to 7.25 with CsOH. After formation of a Gigaohm seal between the patch electrodes and individual Ca_v1.2 stably transfected HEK-293 cells, the cell membrane across the pipette tip was ruptured to assure electrical access to the cell interior. All solutions applied to cells were continuously perfused and maintained at room temperature. As soon as a stable seal could be established, inward calcium currents were measured upon depolarization of the cell membrane from a holding potential of -80 mV to +50 mV in 10 mV increments of 1000 ms duration (Fig. 1). These pulses were followed by a pulse to +10 mV (300 ms) to record the tail current amplitude. The voltage pulses were run at intervals of 10s.

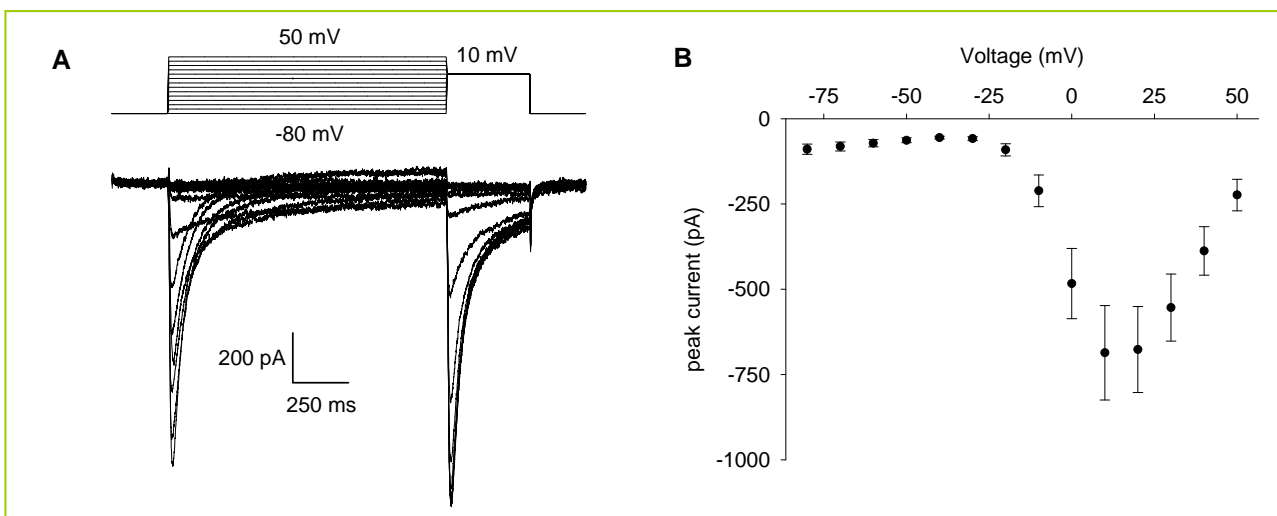


Figure 1: A) Representative current recording of Ca_v1.2 currents. B) Ca_v1.2 IV curve elicited upon depolarization of the cell membrane from holding potential (-80 mV) up to +50 mV in 10 mV increments.

The minimum of the IV curve was found at +20 mV. Based on the IV curve the activation curve was constructed. The $V_{0.5}$ value was determined as 1.34 ± 1.88 mV ($k=6.50 \pm 0.66$), $n=6$ (Figure 2).

To obtain the inactivation curve Ca_v1.2 cells in the whole-cell configuration were depolarized to potentials between -80 mV and +50 mV for 1 s, followed by a test pulse to +10 mV (300 ms). The peak currents were plotted versus the voltage of the depolarizing pre-pulse and fitted with a Boltzmann equation. The $V_{0.5}$ value was determined as -16.40 ± 1.79 mV ($k=7.56 \pm 0.30$), $n=6$. Stimulation frequency: 0.1 Hz (Figure 2).

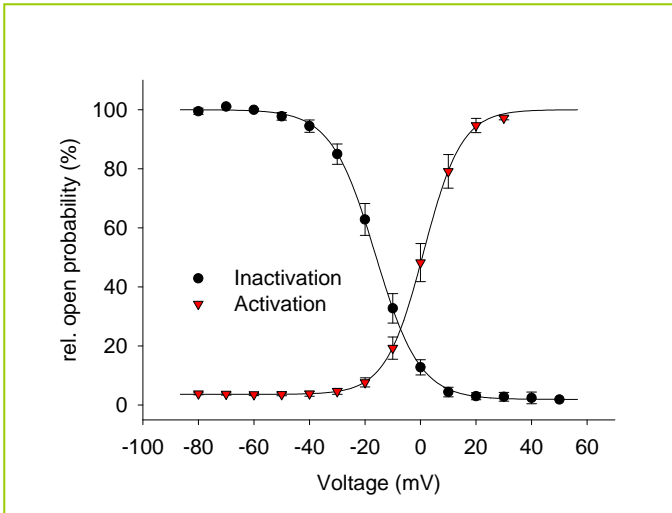


Figure 2: A) Ca_v1.2 activation curve obtained from the IV curve by dividing the current amplitudes by the applied voltage minus the reversal potential. B) Inactivation curve of Ca_v1.2 currents after depolarization for 1 s and a repolarising pulse to 10 mV (100 ms).

2.2 Pharmacological Validation

Ca_v1.2 cells were used in the whole-cell configuration. The effect of known Ca²⁺ channel blockers was tested on these cells. After the current amplitude had stabilized increasing concentrations of the test substances were perfused. Cumulative dose response curves were generated and the IC₅₀ values determined. Between two voltage pulses, the cells were clamped to -80 mV. After a pre-pulse of -50 mV (100 ms) the cells were clamped to 0 mV for 100 ms to open the Ca_v1.2 channels. The time course of the peak current amplitudes were analyzed, normalized to the current amplitude under control conditions and the effect of each tested concentration calculated.

The results are summarized below:

Table 1: IC₅₀ values

Compound	IC ₅₀ value	Hill coefficient
Nifedipine	78.44 nM	0.80
Isradipine	5.99 nM	0.93
Verapamil	8.15 μM	0.74

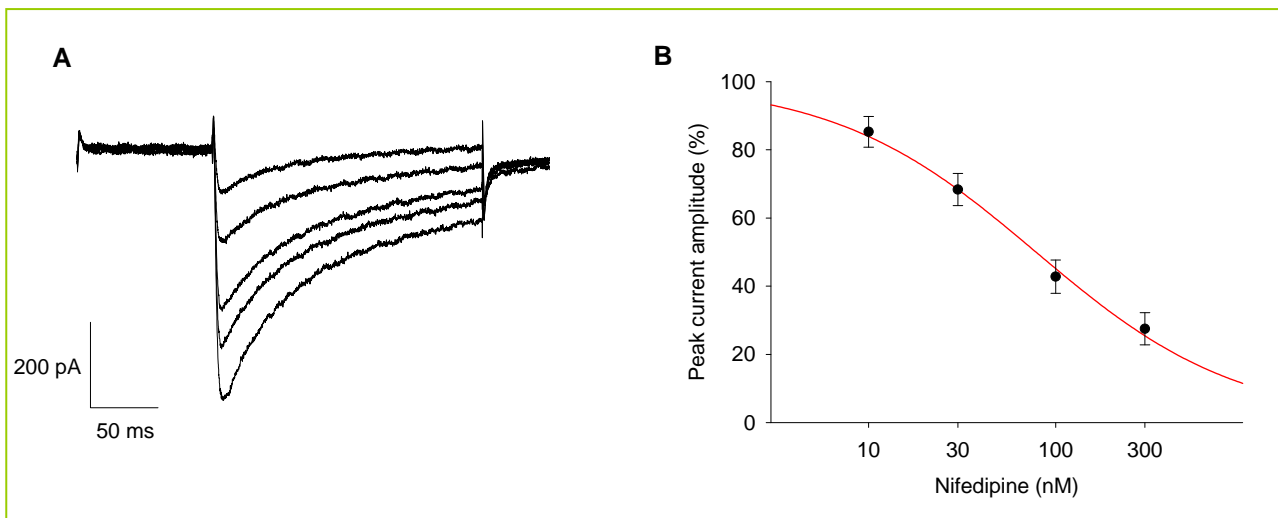


Figure 3: Block of Ca_v1.2 by Nifedipine A) Representative current recording. 0 nM, 10 nM, 30 nM and 100 nM were perfused in increasing concentrations. B) Dose response curve of Ca_v1.2 currents blocked by Nifedipine. The IC₅₀ was determined to be 78.44 nM (Hill coefficient: 0.80).

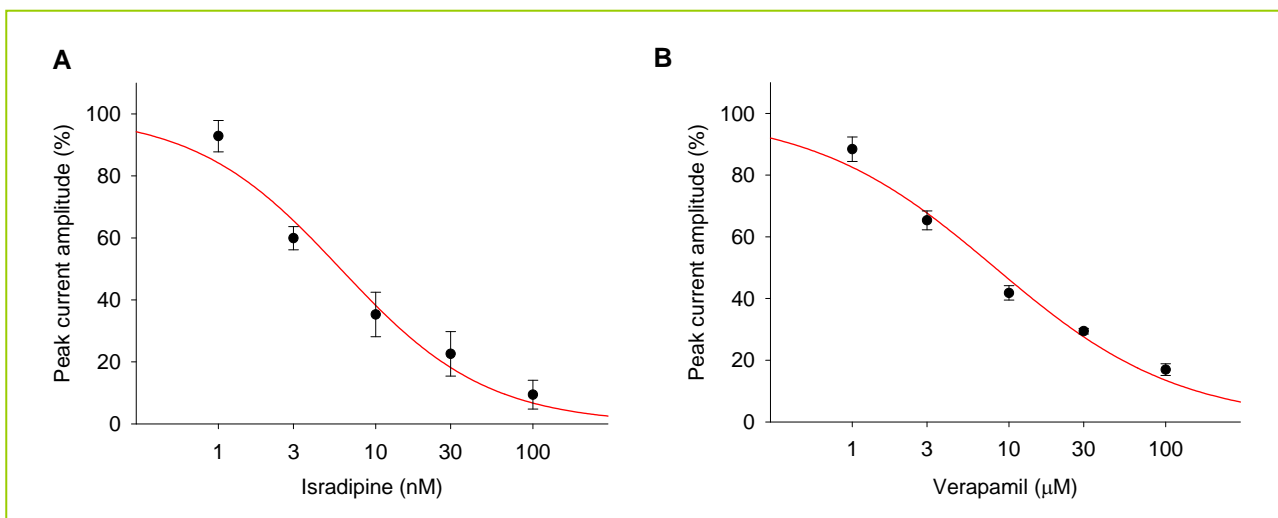


Figure 4: A) Dose response curve of Ca_v1.2 currents blocked by Isradipine. The IC₅₀ was determined to be 5.99 nM (Hill coefficient: 0.93) B) Dose response curve of Ca_v1.2 currents blocked by Verapamil. The IC₅₀ was determined to be 8.15 μM (Hill coefficient: 0.74).

2.3 CiPA Stimulation Protocols

In Autumn 2015 the CiPA (Comprehensive In Vitro Proarrhythmia Assay) consortium suggested two voltage protocols to test drug effects on CaV1.2 channels using patch-clamping. The B'SYS' CaV1.2 cell line is a suitable test system for the CiPA.

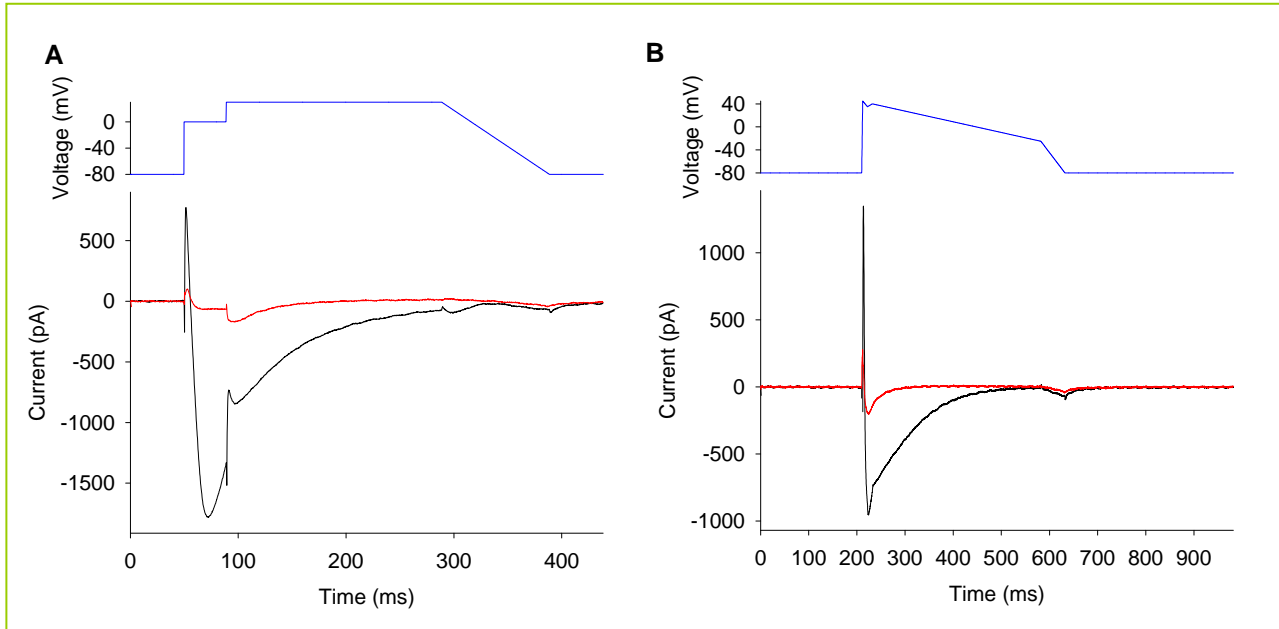


Figure 5: B'SYS Cav1.2 HEK 203 under CiPA voltage protocols: Black: Control conditions, red: 100 μM Verapamil, blue: voltage protocol A) CaV1.2 voltage protocol B) Action potential similar stimulation protocol.

3 CELL CULTURE CONDITIONS

3.1 General

HEK-293 Ca_v1.2/α2δ1/β2 cells are incubated at 37°C C in a humidified atmosphere with 5% CO₂ (rel. humidity > 95%). The cells are continuously maintained and passaged in sterile culture flasks containing DMEM/F12 medium supplemented with 10% foetal bovine serum, 1.0% Penicillin/Streptomycin solution and 100 µg/mL Hygromycin B and 15 µg/mL Blastidicin/ 0.4 µg/mL Puromycin. The HEK-293 Ca_v1.2 cells are passaged at a confluence of about 80%.

- 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% confluence at 1:3 to 1:5 ratio.

3.2 Recommended Complete Medium

- DMEM/F12 with GlutaMAX or Glutamate
- 10% FBS
- 0.9% Penicillin/Streptomycin

3.3 Antibiotics

- HEK-293 Ca_v1.2 clones were selected under 250 µg/mL Hygromycin B / 15 µg/mL Blastidicin / 0.6 µg/mL Puromycin antibiotic pressure.
- To cultivate HEK-293 Ca_v1.2 cells, also a reduced antibiotic pressure (100 µg/mL Hygromycin B / 15 mg/mL Blastidicin/ 0.4 µg/mL Puromycin) can be used.
- To separate HEK-293 Ca_v1.2 cells from untransfected cells, use 250 µg/mL Hygromycin B / 15 µg/mL Blastidicin / 0.4 µg/mL Puromycin.

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

3.4 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 4-6 hours to allow the cells to attach to the bottom of flask.
- Aspirate off the medium and replace with 5 mL complete medium & antibiotics.
- Antibiotics: 100 µg/mL Hygromycin B / 15 mg/mL Blastidicin/ 0.4 µg/mL Puromycin.
- Incubate cells and check them daily until 70% - 80% confluence is reached.

3.5 Splitting Cells

- Remove medium when cells have reached 70% - 80% confluence.
- Wash cells once with 1x PBS to remove excess medium.
- Add 1x Trypsin/EDTA and incubate 30 sec at room temperature.
- Remove Trypsin/EDTA quickly and incubate cells for 2 minutes at 37°C.
- Detach cells, 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.

3.6 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).

3.7 Freezing Cells

- Prepare fresh freezing medium and keep it on ice.
- Cells should have 80% - 90% confluence prior to freezing.
- Remove the complete medium.
- Wash cells once with 1x PBS to remove excess medium.
- Add 1x Trypsin/EDTA and incubate 30 sec at room temperature.
- Remove Trypsin/EDTA quickly and incubate cells for 2 minutes at 37°C.
- Detach cells, add complete medium and pipet up and down to break clumps of cells.
- Pellet cells with centrifuge and carefully aspirate off medium.
- Resuspend cells at a density of approximately 2.0 E+06 cells per ml with fresh freezing medium.
- Aliquot 0.5 mL of cell suspension into each cryovial.
- Incubate cells overnight in a polystyrene box at –80°C.
- Transfer cryovial in liquid nitrogen tank for long-term storage the next morning.

3.8 Inducing Expression of Ca_v1.2

To induce the expression of Ca_v1.2 receptors 2.5 µg/mL Tetracycline has to be added at least 16 h - 24 h before experimentation.

4 Ca_v1.2 SEQUENCE

For some subunits codon optimized sequences were used.

4.1 Ca_v1.2 alpha 1c subunit

MNANAAAGLAPEHIPTPGAALSQAAIDAARQAKLMGSAGNATISTVSSTQRKRQYQYKPKKQGSTTATRPPRALLCLTL
 KNPIRRACISIVEWKPFEEIIILLTIFANCVALAIYIPFPEDDSNATNSNLERVEYLFLLIIFTVEAFLKVIAYGLLFHPNA
 YLRNGWNLLDFIIVVVGFLFSAILEQATKADGANALGGKGAGFDVKALRAFRVLRPLRLVSGVPSLQVVLNSIIKAMVPLL
 HIALLVLFVIIIIYAIIGLELFMGMKHKTCYNQEGEADVPAEDDPSPCALETGHGRQCQNGTVCKPGWDGPKHGITNFDNF
 AFAMLTVFQCITMEGWTDVLYWMDAMGYELPWVYFVSLVIFGSFFVLNLVGLVLSGEFSKEREKAKARGDFQKLREKQQ
 LEEDLKGYLWITQAEDIDPENEDEGMDEEKPRNMSMPTSETESVNTENVAGGDI EGENGARLAHRISKSKFSRYWRRW
 NRFCCRRCRAAVKSNVFWLVIIFLVFLNLTIASEHYNQPNWLTEVQDTANKALLALFTAEMLLKMYSLGLQAYFVSLFN
 RFDVFCVCGGILETILVETKIMSPLGISVLRVLRLLRIFKITYWNSLSNLVASLLNSVRSIASLLLLLFLFIIIFSLLG
 MQLFGGKFNDEMQRTRSTFDNFPQSLLTVFQILTGEDWNSVMYDGMAYGGPSFPGMLVCIYFIIILFICGNII LLNVFL
 AIAVDNLADAESLTSAQKEEEEEKERKKLARTASPEKKQELVEKPAVGESKEEKIELKSITADGESPPATKINMDDLQPN
 ENEDKSPYPNPETTGEDEDEEPEMPVGP RPRLSELHLKEKAVPMPEASAFFIFSSNNRFRQLCHRIVNDTIFTNLI LFF
 ILLSSISLAAEDPVQHTSFRNHILFYFDIVFTTIFTIEIALKMTAYGAFLHKGSFCRNYFNILDLLVSVSLISFGIQSS
 AINVVKILRVLRLRPLRAINRAKGLKHVVQCVFAIRTIIGNIVIVTLLQFMFACIGVQLFKGKLYTCSDSKQTEAEC
 KGNYITYKDGEVDHP I IQPRSWENSKFDFDNVLAAMMALFTVSTFEGWPELLYRSIDSHTEDKGIYNYRVEISIFFIIY
 IIIIAFFMMNIFVGFVIVTFQEQGEQYKNCELDKNQRQCVEYALKARPLRRYIPKNQHQQYKVVVYVNSTYFEYLMFVLI
 LLNTICLAMQHYGQSCLFKIAMNILNMLFTGLFTVEMILKLI AFKPKGYFSDPWNVDFDLIVIGSII DVILSETNPAEHT
 QCSPSMNAEENSRI SITFFRLFRVMRLVKLLSRGEGIRTLTLLWTFIKSFQALPYVALLIVMLFFIYAVIGMQVFGKIALND
 TTEINRNNNFQTFPQAVLLLFRCATGEAWQDIMLACMPGKKCAPESEPSNSTEGETPCGSSFAVFYFISFYMLCAFLIIN
 LRVAVIMDNFDYLTRDWSILGPHHLDEFKRIWAEYDPEAKGRIKHLDVVTLRLRIQPPLGFGKLCPHRVACKRLVSMNMP
 LNSDGTVMFNATLFAVLRALRIKTEGNLEQANEELRAIKKIWKRTSMKLLDQVPPAGDDEVTVGKFYATFLIQEYFR
 KFKKRKEQGLVGKPSQRNALSQAGLRTLHDIGPEIRRAISGDLTAEELDKAMKEAVSAASEDDIFRRAGGLFGNHVSY
 YQSDGRSAFPQTFTTQRPLHINKAGSSQGDTEPSHEKLV DSTFTPSYSSTGSNANINNANNTALGRLPRPAGYPSTVS
 TVEGHGPPSPAIRVQEVAVKLSNRERHVP MCEDELRRDSGSAGTQAHCLLLRKANPSRCHSRESQAAMAGQEETSQD
 ETYEVKMNHDTEACSEPSLLSTEMLSYQDDENRQLTLPEEDKRDIRQSPKRGFLRSASLGRRASFHLECLKRQKDRGGDI
 SQKTVLPLHLVHHQALAVAGLSPLLQRSHSPASFPRPFATPPATPGSRGWPPQVPVTLRLEGVESSEKLNSSFP SIHCGS
 WAETTPGGGGSSAARRVRPVSMLMVPSQAGAPGRQFHGSASSLVEAVLISEGLGQFAQDPKFI EVTTQELADACDMTIEEM
 ESAADNILSGGAPQSPNGALLPFVNCRDAGQDRAGGEEDAGCVRARGRPSEELQDSRVYVSSL

4.2 Ca_v1.2 beta 2 subunit

MLDRRLIAPQTKYIIPGGSADSYTSRPSDSVLSLEEDREAVRREAERQAQAQLEKAKTKPVAFVAVRTNVSYSAAHEDDVP
 VPGMAISFEAKDFLHVKEKFNNDWWIGRLVKEGCEIGFIPSPVKLENMRLQHEQRAKQKGFYSSKSGGNSSSSLGDIVPS
 SRKSTPPSSAIDIDATGLDAEENDIPANHRSPKPSANSVTSPHSKEKRMPPFFKTEHTPPYDVVPSMRPVVLVGP SLKGY
 EVTDMMQALFDLKHREGRISITRVTADISLAKRSVLNPNPKHAI IERSNTRSSLAEVQSEIERIFELARTLQLVVL D
 ADTINHQAQLSKTSLAPIIVYVKISSPKVLQRLIKSRGKSQAKHLNVQMVAAADKLAQCPELFDVILDENQLEDACEHLA
 DYLEAYWKATHPPSSSLPNPLLSRTLATSSLPLSPTLASNSQGSQGDQRTDRSAPIRSASQAESEEPSVEPVKKSQHRSSS
 SAPHHNHRSGTSRGLSRQETFDSETQESRDSAYVEPKEDYSHDHVDHYASHRDHNHRDETHGSSDHRHRESRHRSDVDR
 EQDHNECNKQRSRHKSKDRYCEKDGEVISKRNEAGEWNRDVYIRQ

4.3 Ca_v1.2 alpha2 / delta 1 subunit

MNANAAAGLAPEHIPTPGAALSQAAIDAARQAKLMGSAGNATISTVSSTQRKRQYQYKPKKQGSTTATRPPRALLCLTL
 KNPIRRACISIVEWKPFEEIIILLTIFANCVALAIYIPFPEDDSNATNSNLERVEYLFLLIIFTVEAFLKVIAYGLLFHPNA
 YLRNGWNLLDFIIVVVGFLFSAILEQATKADGANALGGKGAGFDVKALRAFRVLRPLRLVSGVPSLQVVLNSIIKAMVPLL
 HIALLVLFVIIIIYAIIGLELFMGMKHKTCYNQEGEADVPAEDDPSPCALETGHGRQCQNGTVCKPGWDGPKHGITNFDNF
 AFAMLTVFQCITMEGWTDVLYWMDAMGYELPWVYFVSLVIFGSFFVLNLVGLVLSGEFSKEREKAKARGDFQKLREKQQ
 LEEDLKGYLWITQAEDIDPENEDEGMDEEKPRNMSMPTSETESVNTENVAGGDI EGENGARLAHRISKSKFSRYWRRW

NRF CRRK CRAAVKSNV FYWLVIFLVFLNLT LTIA SEHYNQPNWLTEVQDTANKALLALFTAEMLLKMYSLGLQAYFVSLFN
RFDCFVVC GGILETILVETKIMSPLGISVLRVLLRIFK ITRYWNSLSNLVASLLNSVRSIASLLLLLFLFIIIFSLLG
MQLFGGKFNFD EMQTRRSTFDNFPQSLLTVFQILTGEDWNSVMYD GIMAYGGPSFPGMLVCIYFIIILFICGN YILLNVFL
AIAVDNLADAESL TSAQEEEEEEKERKKLARTASPEKKQELVEKPAVGESKEEKIELKSITADGESPPATKINMDDLQPN
ENEDKSPYPNPE TTTGEEDEEEPEMPVGPRRPLSELHLKEKAVPMPEASAFFIFSSNNRFR LQCHRIVNDTI FTNLILFF
ILLSSISLAAEDPVQHTSFRNHILFYFDIVFTTIFTIEIALKMTAYGAFLHKG SFCRNYFNILDLLVVSLSLISFGIQSS
AINVKILRVLRVLRPLRAINRAKGLKHVVQC VFVAIR TIGNIVIVTTLLQFMFACIGVQLFKGKLYTCS DSSKQTEAEC
KGN YITYKDG EVDHP IIQPRSWENSKFDFDNVLAAMMALFTVSTFEGWPELLYRSIDSHTEDKGPIYNYRVEISIFFIIY
IIIIA FFMNIFVGFVIVTFQEQQEQEYKNCELDKNQRQCVEYALKARPLRRYIPKNQH QYKVWYVVN STYFEYLMFVLI
LLNTICLAMQHYGQSCLFKIAMNILNMLFTGLFTVEMILKLI AFKPKGYFSDPWNVDFLIVIGSII DVILSETNPAEHT
QCSPSMNAEENSRISITFFRLFRVMRLVKLLSRGEGIR TLLWTFIKSFQALPYVALLIVMLFFIYAVIGMQVFGKIALND
TTEINRNNNFQTFPQAVLLLFRCATGEAWQDIMLACMPGKKCAPESEPSNSTEGETPCGSSFAVFYFISFYMLCAFLIIN
LFVAVIMDNFDYLTRDWSILGPHHLDEFKRIWAEYDPEAKGR IKHLDVVTLLRRIQPPLGFGKLCPHRVACKRLVSMNMP
LNSDGTVMFNATL FALVRTALRIKTEGNLEQANEELRAI IKKIWKRTSMKLLDQVVP PAGDDEVTVGKFYATFLIQEYFR
KFKKRKEQGLVGKPSQRNALS LQAGLRTLHDIGPEIRRAISGDLTAE EELDKAMKEAVSAASEDDIFRRAGGLFGNHVSY
YQSDGRSAFPQTFTTQRPLHINKAGSSQGDTESPSHEKLV DSTFTPSSYSSTGSNANINNANNTALGR LPRPAGYPSTVS
TVEGHGPPLSPAIRVQEV AWKLSNRERHVPMCEDLELRRDSGSAGTQAHCLLLR KANPSRCHSRESQAAMAGQEETSQD
ETYEVKMNH DTEACSEPSLLSTEMLSYQDDENRQLTLPEEDKRD IRQSPKRGFLRSASLGRRASFHLECLKRQKDRGGDI
SQKTVLPLHLVHHQALAVAGLSPLLQRSHSPASFPRPFATPPATPGSRGWPPQPVPTLRLEGVESSEKLNSSFPSIHCGS
WAETTPGGGSSAARRVRPVS LMVPSQAGAPGRQFHGSASSLVEAVLISEGLGQFAQDPK FIEVTTQELADACDMTIEEM
ESAADNILSGGAPQSPNGALLPFVNCRDAGQDRAGGEEDAGCVRARGRPSEELQDSRVYVSSL

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