ION BIOSCIENCES

Specifications

HEK293-huK$_{ir}$3.1/3.2 Recombinant cell line

Catalog No. C1101

Introduction

G protein-gated inwardly rectifying potassium channels (GIRK) are a large family of inwardly rectifying potassium channels that play important roles in neurophysiology and cardiac physiology. Mammals express four GIRK subunits (K$_{ir}$3.1-K$_{ir}$3.4); the predominant form of GIRK in the brain is a K$_{ir}$3.1/K$_{ir}$3.2 heterotetramer. GIRK channels are opened by the direct binding of the G protein βγ subunit (Gβγ), which is released from the heterotrimeric G protein (Gα$_i$βγ) upon the activation of G protein coupled receptors (GPCRs). GIRK is exclusively associated with G$_{i/o}$ GPCRs such as dopamine receptors, serotonin receptors, muscarinic receptors and opioid receptors. Due to its critical role of regulating cell excitability, GIRK presents a potential therapeutic target for epilepsy, Parkinson's disease, Down's syndrome, pain, drug addiction, and many more pathologies.

Recombinant HEK293-huK$_{ir}$3.1/3.2 cell line express both human KCNJ3 (K$_{ir}$3.1, potassium inwardly rectifying channel subfamily , J member 3, accession number NM_002239) and human KCNJ6 (K$_{ir}$3.2, potassium inwardly rectifying channel subfamily J member 6, accession number NM_002240). K$_{ir}$3.1 is unable to independently form functional channels on the plasma membrane; it forms heterotetrameric channels. By contrast, K$_{ir}$3.2 can form homotetramers and can assemble with other GIRK subunits to form heterotetramers, such as K$_{ir}$3.1/K$_{ir}$3.2. K$_{ir}$3.1/K$_{ir}$3.2 channels have a greater tendency to allow potassium flow into a cell than out of a cell; they modulate many physiological processes, including heart rate in cardiac cells and circuit activity in neuronal cells. Our HEK293-huK$_{ir}$3.1/3.2 cell line is suitable for discovering modulators of GIRK channel activity and screening for agonist/antagonist of various G$_{i/o}$ GPCRs that associate with GIRK channels.

Materials Provided

One vial of HEK293-huK$_{ir}$3.1/3.2 recombinant cells, 2 x 10^6 cells in 1 mL of Bambanker serum free freezing medium.

Storage

Cells are shipped on dry ice and should arrive frozen. To ensure maximum cell viability, store the cell vial in liquid nitrogen immediately upon receipt.

Mycoplasma testing

The cell line has been screened using ATCC PCR-based testing service which covers 60 species of *Mycoplasma, Acholeplasma, Spiroplasma* and *Ureaplasma* including the eight species most likely to afflict cell cultures: *M. arginini, M. fermentans, M. hominis, M.hyorhinis, M. orale, M. pirum, M. salivarium,* and *A. laidlawii.* The absence of Mycoplasma species has been confirmed for each lot. For detailed verification, please see the product certificate of analysis.

Materials Required But Not Supplied

Category	Name	Recommendations
Reagents	Cell culture media	Advanced MEM (ThermoFisher Cat#: 12491013)
	GlutaMax™	GlutaMAX™ Supplement (ThermoFisher Cat#: 35050061)
	Fetal bovine serum (FBS)	Fetal Bovine Serum (Corning Cat#: 35-016-CV)
	Blasticidin	Blasticidin S HCl (ThermoFisher Cat#: A1113903)
	Puromycin	Puromycin Dihydrochloride (ThermoFisher Cat#: A1113803)
	TrypLE™	TrypLE™ Express Enzyme (ThermoFisher Cat#: 12605028)
	Phosphate buffered saline	PBS, pH 7.4 (ThermoFisher Cat#: 10010023)
	Freezing media	Bambanker Serum Free Cell Freezing Medium
Equipment	Single and multichannel micropipettes and pipette tips	
	50 mL and 15 mL conical centrifuge tubes	
	1.5 mL microtubes	
	Tissue culture disposable pipettes (1 mL-25 mL) and tissue culture flasks (e.g. T75 flask)	
	0.2 µm filter unit for medium sterilization	
	Cryovials for freezing cells	
	Automated Cell Counter (or Hemocytometer)	
	Humidified tissue culture incubator (37°C and 5% CO_2)	

Cell Culture Protocol

A. Cell culture media

1. Make thawing medium by combining 25 mL of FBS, 5 mL of 100X GlutaMax with 470 mL 1X Advance MEM media for the final concentration of 5% FBS and 1X GlutaMax.
2. To make complete culture medium, add 250 µL of 10 mg/mL Blasticidin, and 150 µL of 10 mg/mL Puromycin into 500 mL of medium from **step 1** for the final concentrations of 5 µg/mL Blasticidin and 3 µg/mL Puromycin.
3. (Optional) Sterilize all media using 0.2 µm filter.
4. Pre-warm all media in a 37°C water bath prior to use.

B. Thawing cells

1. Aliquot 8 mL of thawing medium in a 15 mL conical centrifuge tube.
2. Place the cryovial in a 37°C water bath briefly, until only small ice crystals remain and the cell pellet is almost completely thawed. The thawing time typically ranges from 2 to 3 minutes. DO NOT vortex freshly thawed cells.
3. Spray and wipe the external surface of the cryovial with 70% ethanol. Transfer the vial to a biosafety cabinet.

Cell Culture Protocol Cont.

4. Gently add the cells into the pre-filled 15 mL conical tube dropwise.
5. Rinse the cryovial with 1 mL of cell suspension to maximize cell recovery and add it back into the 15 mL conical tube.
6. Centrifuge the 15 mL conical tube containing cells at 500xg for 5 min at 25°C.
7. Add 14 mL of complete cell culture medium into a T75 flask.
8. After centrifugation, carefully aspirate the supernatant without disturbing the cell pellet.
9. Gently resuspend the cell pellet with 1 mL of complete cell culture medium.
10. Transfer the cell suspension to the T75 flask and gently swirl the flask to distribute the cells evenly in solution.
11. Incubate the flask in a 37°C and 5% CO2 humidified cell culture incubator.
12. Maintain the cells in culture by exchanging culture media every 2-3 days, until they reach >80% confluency in a T75.

C. Cell passage

1. Remove the T75 flask from the tissue culture incubator and place it in a biosafety cabinet.
2. Gently aspirate the media from the T75 flask.
3. Add 15 mL of PBS into the T75 flask, and gently rock the flask back and forth to rinse the cells.
4. Gently aspirate PBS from the flask.
5. Add 3 mL of pre-warmed TrypLE to the flask. Gently rock the flask back and forth to ensure that the flask is uniformly covered with TrypLE.
6. Incubate the flask at 37°C and 5% CO_2 until the cells have detached. Usually it will take 8 - 10 min.
7. Remove the flask from the incubator and observe the cells under a microscope to confirm cell detachment. If necessary, gently tap the edge of the flask to detach cells from the surface.
8. Add 10 mL of pre-warmed complete cell culture media to the detached cells in the flask.
9. Transfer cell suspension into a 15 mL conical tube.
10. Centrifuge the 15 mL conical tube containing cells at 500xg for 5 min at 25°C.
11. After centrifugation, carefully aspirate the supernatant without disturb the cell pellet.
12. Resuspend the cell pellet with 5 mL of complete cell culture medium. Pipette the cell suspension up and down several times to generate a single cell suspension without any clumps.
13. Take a small aliquot of the cell suspension (~20 µL) for cell counting, and determine the number of cells and volume required to seed into a T75 flask. HEK293-huK_{ir}3.1/3.2 cell doubling time is ~36h. A confluent T75 flask normally yields ~10x10^6 cells. We recommend to seed 1 X 10^6 cells for a T75 flask.
14. Add 15 mL of complete cell culture medium into a new T75 flask, followed by addition of the appropriate volume of cell suspension. Transfer the flask to a tissue culture incubator, and incubate the cells at 37°C and 5%CO_2.

Cell Culture Protocol Cont.

D. Cryopreservation

1. Harvest cells according to the description in **section C** Cell Passage **step 1 - 9**.
2. Set aside a small fraction of the suspended cells (100 µL or less) in a separate tube for cell counting.
3. Count cells, calculate the concentration of cells and the total number of cells in the original 15 mL conical tube.
4. Centrifuge the 15 mL conical tube containing cells at 500xg for 5 min at 25°C.
5. After centrifugation, discard the supernatant, being careful not to disturb the cell pellet.
6. Based on the total cell number calculated in **Step 3**, resuspend the cells to the desired concentration (e.g. 2.0 x 10^6 cells/mL) in Bambanker serum free cell freezing medium.
7. Aliquot 1 mL of the cell suspension into each of the labeled 2 mL cryovials. Seal the cryovials tightly.
8. Freeze cells in a -80°C freezer at a controlled rate of -1°C/minute overnight in a commercially-available freezing container.
9. The following day, transfer the vials into the vapor phase of liquid nitrogen for long-term storage.

Functional Validation

The function of $K_{ir}3.1/3.2$ channels were validated by GIRK potassium channel activator ML297 (VU0456810) (Figure 1) and VU551 (VU0529331) (Figure 2). In addition, $G_{i/o}$ GPCRs were co-expressed into HEK293-huK$_{ir}$3.1/3.2 cells; GPCR agonists activated $K_{ir}3.1/3.2$ channels, resulting in an increased Tl^+ influx detected using $G_{i/o}$ GPCR-GIRK thallium flux assay (Figure 3). HEK293-huK$_{ir}$3.1/3.2 cells co-expressing dopamine D2 receptors showed distinct signal increases upon activation by dopamine in a HTS assay with Z' factor = 0.55 (Figure 4).

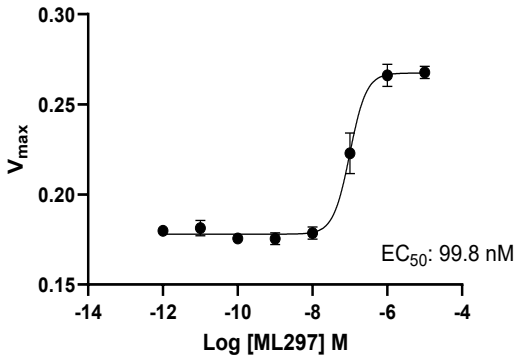

Figure 1. ML297 Dose Response Curve. Thallium flux assay was performed using FlexStation 3 kinetic plate reader with excitation wavelength 490 nm and emission wavelength 520 nm. HEK293-$K_{ir}3.1/3.2$ cells were treated with potassium channel activator ML297 at the highest concentration of 10 µM. Dose response curves generated an EC_{50}: 100 nM.

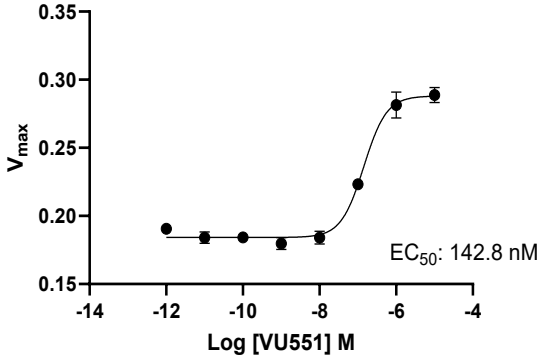

Figure 2. VU551 Dose Response Curve. Thallium flux assay was performed using FlexStation 3 kinetic plate reader with excitation wavelength 490 nm and emission wavelength 520 nm. HEK293-$K_{ir}3.1/3.2$ cells were treated with potassium channel activator VU551 at the highest concentration of 10 µM. Dose response curves generated an EC_{50}: 143 nM.

Functional Validation Count.

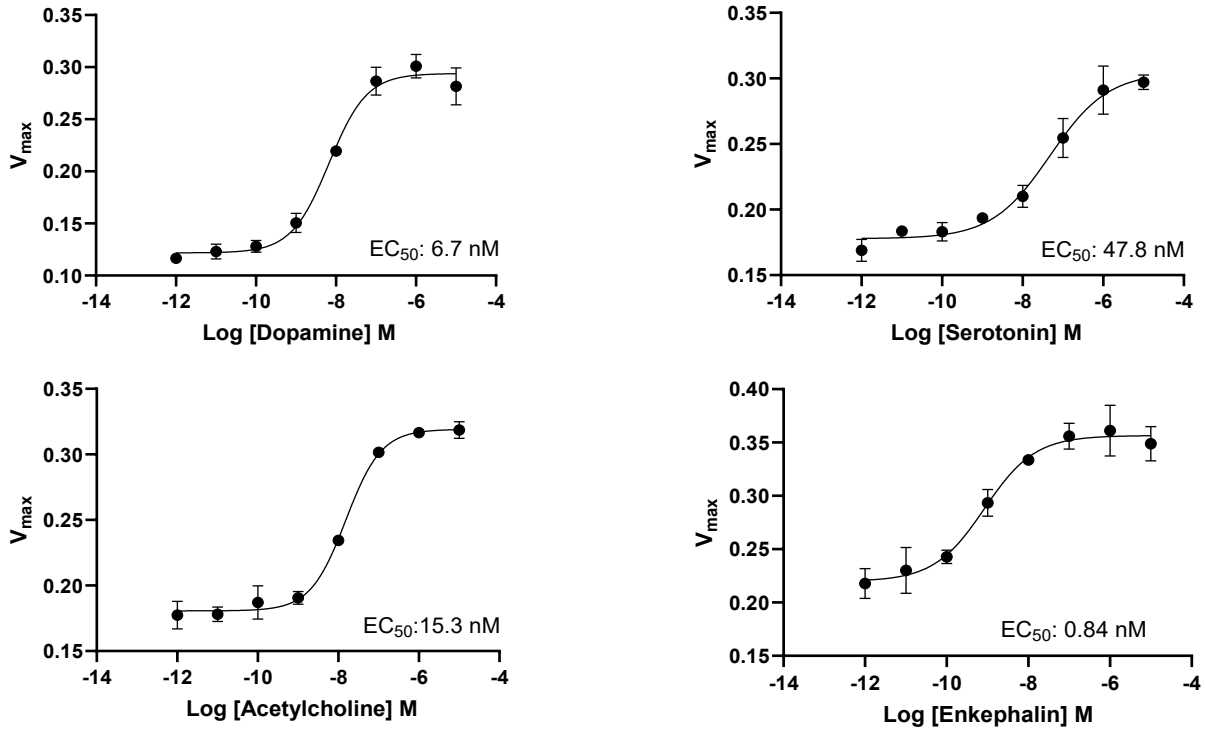

Figure 3. 96-well $G_{i/o}$ GPCR-GIRK Thallium Flux Assay Dose Response Curves. Dopamine D2 receptor, Serotonin 1A receptor, Muscarinic M2 receptor, and delta opioid receptor were transduced in HEK293-huK$_{ir}$3.1/3.2 cells using the optimized amount of transduction conditions for 24h. Thallium flux assay was performed using a FlexStation 3 kinetic plate reader with excitation wavelength 490 nm and emission wavelength 520 nm. Dopamine, Serotonin, Acetylcholine, and Enkephalin were tested with the highest concentration of 10 µM. Dose response curves generated dopamine EC_{50}: 6.7 nM, serotonin EC_{50}: 47.8 nM, acetylcholine EC_{50}: 15.3 nM, and Enkephalin EC_{50}: 0.84 nM.

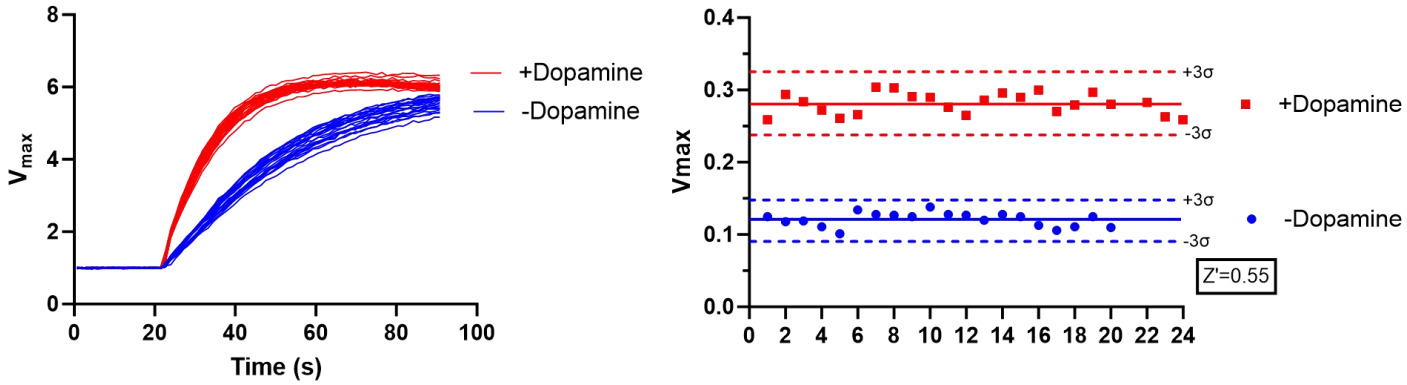

Figure 4. 96-well $G_{i/o}$ GPCR-GIRK Thallium Flux Assay Z' Factor Analysis. Dopamine D2 receptor was transduced in HEK293-huK$_{ir}$3.1/3.2 cells using the optimized transduction conditions for 24h. Thallium Flux assay was performed using FlexStation 3 kinetic plate reader with excitation wavelength 490 nm and emission wavelength 520 nm. For positive control group 10 µM of dopamine was used to activate dopamine D2 receptor; for negative control group buffer alone was added to the plate. Normalized kinetic data showed a clear distinction between positive and negative control groups. The calcualted Z' factor is 0.55 indicating a high quality screening assay.

Vector and Sequence

Vector description

1. Mammalian Gene Expression Vector, pRP[Exp]-Bsd-CAG>hKCNJ3[NM_002239.4]
2. Mammalian Gene Expression Vector, pPB[Exp]-Puro-CAG>hKCNJ6[NM_002240.5]

hKCNJ3 sequence (NM_022039.4)

MSALRRKFGDDYQVVTTSSSGSGLQPQGPGQDPQQQLVPKKKRQRFVDKNGRCNVQHGNLGSETSRYLSDLFTTLVDLKWRWNLFIFIL
TYTVAWLFMASMWWVIAYTRGDLNKAHVGNYTPCVANVYNFPSAFLFFIETEATIGYGYRYITDKCPEGIILFLFQSILGSIVDAFLIGCMFI
KMSQPKKRAETLMFSEHAVISMRDGKLTLMFRVGNLRNSHMVSAQIRCKLLKSRQTPEGEFLPLDQLELDVGFSTGADQLFLVSPLTICH
VIDAKSPFYDLSQRSMQTEQFEIVVILEGIVETTGMTCQARTSYTEDEVLWGHRFFPVISLEEGFFKVDYSQFHATFEVPTPPYSVKEQEE
MLLMSSPLIAPAITNSKERHNSVECLDGLDDITTKLPSKLQKITGREDFPKKLLRMSSTTSEKAYSLGDLPMKLQRISSVPGNSEEKLVSKT
TKMLSDPMSQSVADLPPKLQKMAGGAARMEGNLPAKLRKMNSDRFT

hKCNJ6 sequence (NM_002240.5)

MAKLTESMTNVLEGDSMDQDVESPVAIHQPKLPKQARDDLPRHISRDRTKRKIQRYVRKDGKCNVHHGNVRETYRYLTDIFTTLVDLKW
RFNLLIFVMVYTVTWLFFGMIWWLIAYIRGDMDHIEDPSWTPCVTNLNGFVSAFLFSIETETTIGYGYRVITDKCPEGIILLLIQSVLGSIVNA
FMVGCMFVKISQPKKRAETLVFSTHAVISMRDGKLCLMFRVGDLRNSHIVEASIRAKLIKSKQTSEGEFIPLNQTDINVGYYTGDDRLFLVS
PLIISHEINQQSPFWEISKAQLPKEELEIVVILEGMVEATGMTCQARSSYITSEILWGYRFTPVLTLEDGFYEVDYNSFHETYETSTPSLSAK
ELAELASRAELPLSWSVSSKLNQHAELETEEEEKNLEEQTERNGDVANLENESKV

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